Pharmacoproteomic Study of Three Different Chondroitin Sulfate Compounds on Intracellular and Extracellular Human Chondrocyte Proteomes

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Chondroitin sulfate (CS) is a symptomatic slow acting drug for osteoarthritis (OA) widely used for the treatment of this highly prevalent disease, characterized by articular cartilage degradation. However, little is known about its mechanism of action, and recent large scale clinical trials have reported variable results on OA symptoms. Herein, we aimed to study the modulations in the intracellular proteome and the secretome of human articular cartilage cells (chondrocytes) treated with three different CS compounds, with different origin or purity, by two complementary proteomic approaches. Osteoarthritic cells were treated with 200 μg/ml of each brand of CS. Quantitative proteomics experiments were carried out by the DIGE and stable isotope labeling with amino acids in cell culture (SILAC) techniques, followed by LC-MALDI-MS/MS analysis. The DIGE study, carried out on chondrocyte whole cell extracts, led to the detection of 46 spots that were differential between conditions in our study: 27 were modulated by CS1, 4 were modulated by CS2, and 15 were modulated by CS3. The SILAC experiment, carried out on the subset of chondrocyte-secreted proteins, allowed us to identify 104 different proteins. Most of them were extracellular matrix components, and 21 were modulated by CS1, 13 were modulated by CS2, and 9 were modulated by CS3. Each of the studied compounds induces a characteristic protein profile in OA chondrocytes. CS1 displayed the widest effect but increased the mitochondrial superoxide dismutase, the cartilage oligomeric matrix protein, and some catabolic or inflammatory factors like interstitial collagenase, stromelysin-1, and pentraxin-related protein. CS2 and CS3, on the other hand, increased a number of structural proteins, growth factors, and extracellular matrix proteins. Our study shows how, from the three CS compounds tested, CS1 induces the activation of inflammatory and catabolic pathways, whereas CS2 and CS3 induce an anti-inflammatory and anabolic response. The data presented emphasize the importance of employing high quality CS compounds, supported by controlled clinical trials, in the therapy of OA. Finally, the present work exemplifies the usefulness of proteomic approaches in pharmacological studies.

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Osteoarthritis (OA)1 is the most common rheumatic disease, affecting more than 100 million individuals worldwide. It is mainly characterized by articular cartilage degradation. Despite its high prevalence, there are no available medications to date capable to halt cartilage degeneration or to repair damaged cartilage. The drugs currently available to treat OA are predominantly directed toward the symptomatic relief of pain and inflammation, but they do little to reduce joint destruction. The lack of effective therapies that prevent, inhibit, or reverse the progress of OA often leaves the only option of surgical interventions (1). The effective prevention of the structural damage is a key objective of new therapeutic approaches (2). Multiple compounds, drugs, and nutraceuticals have been investigated for their positive in vitro and/or in vivo effects (3–5). Among pharmacological treatments, symptomatic slow acting drugs for osteoarthritis have been largely studied over the last decade. The use of chondroitin sulfate (CS), alone or in combination with glucosamine sulfate (GS), is globally ris-
ing. CS is a glycosaminoglycan and a major component of the extracellular matrix of many connective tissues, including cartilage. Although its mechanisms of action remain unclear, *in vitro* experiments suggest that CS stimulates the production of proteoglycans by chondrocytes (6, 7) and synoviocytes (6) and inhibits the expression of IL-1-induced metalloproteinases and prostaglandin E2 (7–9), thus preventing cartilage damage. At the same time, CS has shown to have a positive effect on some of the OA-related pathological processes involving the synovial tissue and subchondral bone (7).

The CS employed in scientific studies is generally derived from animal sources by extraction and purification processes and is mainly obtained from bovine, porcine, chicken, or marine cartilage. From these, bovine CS is the most often used *in vitro* and in clinical trials (8). Although beneficial effects of orally administrated CS in OA patients have been reported (14–16), caution should be exercised in the study or use of different CS formulations, because the species or tissue of origin could result in great differences in CS structural organization or disaccharide composition. Probably for this reason, recent meta-analysis (9) and large scale clinical trials (10) have demonstrated variable effects on OA symptoms, yielding conflicting results.

To unravel the molecular mechanisms driven by CS, we recently performed a gel-based proteomic study using normal chondrocytes stimulated with IL-1β (*an in vitro* model of inflammation) and treated with CS alone or in combination with GS (14). However, it should be noted that the quality of the CS formulations (*i.e.* those available as dietary supplements in some countries, such as the United States) is poorly regulated, and this may affect the therapeutic outcome because of differences in molecular composition, tissue of origin, purity, and production/purification processes. Recently, Tat et al. (8) compared CS from three different manufacturers and different sources and measured by ELISA the protein modulations of factors including prostaglandin E2, IL-6, and MMP-1. They concluded that not all brands are the same or act similarly: some may be highly efficacious, whereas others may show barely any effect or even induce unwanted effects. In the present work, we have employed two different quantitative proteomics techniques, DIGE and SILAC, to investigate deeper on the diverse biological outcomes of these different CS compounds, by carrying out comparative analyses on chondrocyte intracellular and extracellular proteomes.

**EXPERIMENTAL PROCEDURES**

*Reagents, Chemicals, and Antibodies—* Culture media and fetal bovine serum (FBS) were from Invitrogen. Culture flasks and plates were purchased from Costar (Cambridge, MA). DIGE materials (IPG buffer and strips and Cy dyes) were from GE Healthcare. SILAC materials (SILAC Dulbecco’s modified Eagle’s medium, dialyzed FBS, and amino acids) were from Silantes (Munich, Germany). Unless indicated, all other chemicals and enzymes were obtained from Sigma-Aldrich. Monoclonal antibody against human manganese-superoxide dismutase (SOD2) was from BD Biosciences. The correspond-
supplemented with 10% dialyzed FBS, 4.5 g/liter glucose, 2 mM CO's modified Eagle's medium lacking arginine and lysine and drocytes were recovered and plated at low density in SILAC Dulbec-

**Image Acquisition and DIGE Data Analysis—** Semi-automated image analysis was performed with Progenesis SameSpots V3.2 software (Nonlinear Dynamics). Image quality control was first performed to identify saturated spots. Multiplexed analysis was selected for DIGE experiments, and a representative gel image was chosen as reference. Spots were detected, and their normalized volumes were ranked on the basis of analysis of variance p values, fold changes, and statistical power (which reflect our confidence in the ability of the experimental data to find the differences that do actually exist).

**MS Analysis of the Gel Spots—** The gel spots of interest were manually excised and transferred to microcentrifuge tubes. Samples selected for analysis were in-gel reduced, alkylated, and digested with trypsin according to the method of Sechi and Chait (14). The samples were analyzed using the MALDI-TOF/TOF mass spectrometer 4800 Proteomics Analyzer (ABSCIEX, Framingham, MA) and 4000 Series Explorer™ software (ABSCIEX, Data Explorer version 4.2) (ABSCIEX) was used for spectra analyses and generating peak picking lists. All of the mass spectra were internally calibrated using autoproteolytic trypsin fragments and externally calibrated using a standard peptide mixture (Sigma-Aldrich). TOF/TOF fragmentation spectra were acquired by selecting the 10 most abundant ions of each MALDI-TOF peptide mass map (excluding trypsin autopeptides and other known background ions).

**Database Search—** The monoisotopic peptide mass fingerprinting data obtained by MS and the amino acid sequence tag obtained from each peptide fragmentation in MS/MS analyses were used to search for protein candidates using Mascot version 2.2 from Matrix Science. Peak intensity was used to select up to 50 peaks/spectr for peptide mass fingerprinting and 50 peaks/predmissor for MS/MS identification. Tryptic autolytic fragments, keratin, and matrix-derived peaks were removed from the data set used for the database search. The searches for peptide mass fingerprints and tandem MS spectra were performed in the UniProt knowledgebase (2010_09 release version, August 10, 2010), by searching in the UniProtKB/Swiss-Prot database, containing 519,348 entries. Fixed and variable modifications were considered (Cys as S-carbamidomethyl derivate and Met as oxidized methionine, respectively), allowing one trypsin missed cleavage site and a mass tolerance of 50 ppm. For MS/MS identifications, a precursor tolerance of 0.02 Da was used. Identifications were accepted as positive when at least five peptides matched and at least 20% of the peptide coverage of the theoretical sequences matched within a mass accuracy of 50 or 25 ppm with internal calibration. Probability scores were significant at p < 0.01 for all matches. The intracellular localization of the identified proteins was predicted from the amino acid sequence using the PSORT II program.

**Chondrocyte Metabolic Labeling and Differential Treatment of SILAC Cell Populations—** For SILAC experiments, freshly isolated chondrocytes were recovered and plated at low density in SILAC Dulbecco's modified Eagle's medium lacking arginine and lysine and supplemented with 10% dialyzed FBS, 4.5 g/liter glucose, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. In the case of light media, standard L-lysine and L-arginine were added, whereas in the heavy media, isotope-labeled L-lysine (13C6) and iso-
tope-labeled L-arginine (15N4) were used. Cell expansion was carried out as described previously by our group (15). Chondrocytes were used at week 3 in primary culture (P1) when 100% of labeling was reached. The cells were washed thoroughly to remove abundant serum proteins and incubated for 48 h in serum-free medium supple-
mented with 200 µg/ml of CS1, CS2, or CS3.

**Collection and Preparation of Conditioned Media for Analysis by LC/MS—** Conditioned media obtained from three different donors were analyzed independently. In addition, off-gel measurements were performed in duplicate to assess the technical reproducibility of the LC-MS set-up. Conditioned media were collected, centrifuged, and filtered using a 0.2-µm filter to ensure removal of any dead cells. Proteins in each individual medium were precipitated with 0.02% sodium deoxycholate for 10 min and then with 10% (v/v) TCA over-

**NanoLC-MALDI-MS Analysis—** Extracted peptide mixtures were desalted and concentrated through a C18 microcolumn (NUTip, Gly-
gen) and finally eluted from the C18 bed using 70% ACN, 0.1% TFA. The organic component was removed by evaporating in a vacuum centrifuge, and the peptides were resuspended in 2% ACN, 0.1% TFA. 5 µl were injected into a reversed phase column (Integrafit C18, ProteopepTM II; New Objective) for nano-flow LC analysis, using a Tempo nanoLC (Eksigent) equipped with a SunCollect MALDI Spotter/Micro-Fraction Collector (SunChrom Wissenschaftliche Geräte GmbH). LC eluate microfractions were mixed with MALDI matrix (3 mg/ml α-cyano-4-hydroxyquinamic acid in 70% ACN and 0.1% TFA, containing 10 fmol/µl angiotensin as an internal standard) and deposited onto an Opti-TOF LC MALDI target plate (1534-spot format, ABSCIEX) with a speed of one spot/15 s. Mass spectrometry analysis was performed on a 4800 MALDI-TOF/TOF instrument (ABSciex) with a 200 Hz repetition rate (Nd:YAG laser). MS full scan spectra were acquired from 800 to 4000 m/z. A total of 1500 laser shots were accumulated for each TOF-MS spectrum at an optimized fixed laser setting. Tandem MS mode was operated with 1 kV of collision energy with collision-induced dissociation (CID) gas (air) over a range of 60 m/z to −20 m/z of the precursor mass value. The precursor mass window was 300 ppm full width at half maximum (fwhm) in reflective mode. A minimum of 800 and a maximum of 1500 laser shots were accumulated with laser stop conditions set at 10 product ion peaks with signal-to-noise ratios of >100 at an optimized, fixed laser setting with metastable suppressor option on. Data-dependent tandem MS settings included acquisition of up to 20 most intense ion signals per spot. If two or more consecutive spots in an LC run with precursor m/z were within 200 ppm tolerance, the spot with the maximum signal-to-noise ratio was subjected to tandem MS analysis.

**Data Analysis of the NanoLC-MS Experiments—** Identification of proteins and relative quantification of their abundance were per-

**Experimental Data to Find the Differences That Do Actually Exist**

**Organic Component**

**MALDI-TOF/TOF**

**Experimental Data to Find the Differences That Do Actually Exist**
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Fig. 1. DIGE experimental design. Chondrocytes were obtained from three pathological OA cartilages and primary cultured. After the first passage, the cells were treated with 200 μg/ml of each brand of CS for 48 h. Untreated cells were used as control (CTL). Chondrocytic proteins were extracted and labeled with the corresponding Cy dyes, following the scheme shown in the table. The samples were then mixed and resolved on six independent DIGE gels. Three fluorescence images were obtained from each gel and subjected to image analysis using SameSpots software.

cystein alkylation with iodoacetamide, urea denaturation, one missed cleavage allowed in trypsin digestion, and focus on biological modifications. Only proteins identified with at least 95% confidence, or a Prot Score (ProteinPilot™ software protein confidence measure) of at least 1.3 were reported (supplemental Table 2). The data were normalized for loading error by bias correction. The PSPEP program was used independently to calculate false discovery rates. Searches against a concatenated database containing both forward and reversed sequences allowed the false discovery rate to be kept at 1%.

Western Blot Analysis—Two-dimensional Western blot analyses were performed following standard procedures. Briefly, 20 μg of secreted proteins were loaded onto 7-cm pH 3–11 NL IPG strips for first dimension separation, and then resolved using standard 10% SDS-PAGE. The separated proteins were then transferred to PVDF membranes (Immobilon P, Millipore Co., Bedford, MA) by electroblohtting and probed with specific antibody against SOD2. Immunoreactive bands were detected by chemiluminescence using corresponding horseradish peroxidase-conjugated secondary antibodies and ECL detection reagents (GE Healthcare) and then digitized using the LAS 3000 image analyzer. Equivalent loadings were verified by Ponceau Red staining after transference.

Statistical Analysis—Each experiment was repeated at least three times. The statistical significance of the differences between mean values was determined using a two-tailed t test, considering significant p values ≤ 0.05. In the proteomic analyses, normalization tools and statistical package from SameSpots and ProteinPilot software were employed. We considered statistically significant only those changes with a p value of ≤0.05 and a ratio ≥1.2 (or ≤0.83), while exhibiting high statistic power (>0.8, in the SameSpots analysis) or low error factor of the quantification (<2, in the ProteinPilot analysis). Where appropriate, the results are expressed as the means ± S.E.

RESULTS

Analysis of the Effect of Different CS Compounds on Chondrocytes Intracellular Proteins—To assess the influence of three different types of CS on the intracellular proteome of human osteoarthritic chondrocytes, we compared four different conditions: cells before treatment (control), CS1-treated cells, CS2-treated cells, and CS3-treated cells. Whole cell extracts were collected 48 h after CS addition, according to a protocol previously set up by our group (11, 16). These samples were then subjected to a six-plex two-dimensional DIGE analysis, resulting in three individual images from each gel (each one corresponding to Cy2-, Cy3-, and Cy5-labeled samples). Fig. 1 describes the experimental procedure followed. The 18 gel images were analyzed using SameSpots software, which allowed the detection of an average of 1500 protein spots on each image. Then interimage spot matching was carried out automatically by the software. In this step, an average of 1200 spots were matched between the gels, and the average relative abundances were calculated. We considered changes within a 95% confidence interval (p ≤ 0.05) and standardized average spot volume ratios exceeding 1.2 with a statistical power of >0.8, based on previous experience by our group (13, 17, 18). This analysis resulted in the detection of 46 spots significantly and reproducibly altered: 27 modulated by CS1, 4 by CS2, and 15 by CS3. These spots are depicted on a DIGE gel image shown in Fig. 2.

Identification of Intracellular Proteins Differentially Modulated by CS—The 46 altered spots were excised from the gels and analyzed by MALDI-TOF and MALDI-TOF/TOF MS. The proteins present in 28 of these spots could be identified and are listed in Table I. We found 10 redundancies caused by post-translational modifications or proteolysis; thus only 18 different proteins were identified as significantly modulated by CS treatment. A database searches was carried out to analyze the predicted subcellular localization of all these proteins and to assign them into different functional groups. As shown in Fig. 3A, most of the identified proteins (52%) were predicted as cytoplasmic, and an additional 30% were described as either associated with the cytoskeleton (11%), located in the nucleus (19%), or located in other subcellular organelles, such as the endoplasmic reticulum (4%) or the mitochondrion (7%). Regarding their biological role, most of these proteins are involved in metabolism and energy production processes (33%) or protein synthesis, folding, and degradation (22%). Some of them are structural pro-
teins (17%) or proteins involved in cell adhesion (11%) (Fig. 3B).

The quantitative comparison between the effect of each CS compound and the basal condition (untreated OA chondrocytes) revealed the alteration of 22 spots in the gels, corresponding to 13 different proteins that are listed in Table II. CS1 modulated 13 spots corresponding to six different proteins: three were decreased (GANAB, VINC (vinculin), and LMNA (lamin A/C)), and three were increased (NAMPT (nicotinamide phosphoribosyltransferase), NNMT (nicotinamide-N-methyltransferase), and SODM). CS2 only modulated the expression of two proteins, both decreased: LEG3 (galec-tin-3) and PSB1 (proteasome subunit β type-1). Finally, CS3 modulated 10 spots corresponding to eight different proteins: three were increased (CALD1 (caldesmon 1), HSP7C (heat shock cognate 71-kDa protein), and VIME (vimentin)), and five were decreased (GANAB, NAMPT, DHE3 (glutamate dehydrogenase 1, mitochondrial), PSB1, and PRDX4 (peroxiredoxin-4)).

The number of altered proteins rose when comparing the effect of CS from different origin (Table III, porcine CS1 versus bovine CS2 or CS3). In this case, eight spots corresponding to six distinct proteins resulted differentially modulated by CS treatment: four were decreased by CS2 (or increased by CS3) and two were increased by CS2 (or decreased by CS3).

Verification of SOD2 Increase by CS1—Mitochondrial SOD2 (or SODM) is a protein previously described as related with the OA process (19). This protein was found in our previous pharmacoproteomic work as decreased by CS, alone or in combination with GS, in normal chondrocytes stimulated with IL-1β (13). Inversely, we found it increased in OA chondrocytes treated with CS1 in the present study (Table II): the DIGE analysis allowed the identification of three SOD2 protein isoforms (corresponding to spots 1214, 1215, and 1217), all of them exhibiting a CS1-dependent increase higher than 2-fold (Table I). Therefore, we performed additional two-dimensional Western blot analyses on chondrocyte protein extracts (n = 4) to verify this increased expression. As shown in Fig. 4, an increase in SOD2 levels was evident for all protein isoforms.

Secretome Analysis by SILAC Allows the Identification of Several Extracellular Matrix Proteins Modulated by CS—Given the key role of chondrocytes in extracellular matrix synthesis and turnover, and also the importance of these mechanisms for tissue maintenance (which is disturbed in OA and other joint diseases), we pursued examination of the effect of the three CS compounds in the subset of proteins secreted by chondrocytes (secretome). With this aim, supernatants from SILAC-labeled chondrocytes, untreated and treated with CS,
### Table I

Intracellular proteins identified by two-dimensional DIGE-MS as differentially modulated in CS-treated OA chondrocytes

| Spot number | Accession number | Symbol | Protein name | Loc | Cellular role | Mr/pI | Number of peptides | Cov (%) | Sequence tag |
|-------------|------------------|--------|--------------|-----|---------------|-------|-------------------|--------|--------------|
| 257         | Q14697           | GANAB  | Neutral α-glucosidase AB | ER/Golgi | Protein folding | 107.26/5.74 | 8 | 7 |
| 393         | P18206           | VINC   | Vinculin | Cyt/CK | Cell-matrix adhesion | 124.29/5.50 | 14 | 9 |
| 447         | Q05682           | CALD1  | Caldesmon | Cyt/CK | Cell motion | 93.23/5.62 | 7 | 8 |
| 427         | Q05682           | CALD1  | Caldesmon | Cyt/CK | Cell motion | 93.23/5.62 | 7 | 8 |
| 512         | P11142           | HSP7C  | Heat shock cognate 71-kDa protein | Cyt | Protein folding | 71.08/5.37 | 7 | 11 |
| 490         | P02545           | LMNA   | Lamin-A/C | Nucl | Nuclear assembly | 74.38/6.57 | 16 | 23 |
| 491         | P02545           | LMNA   | Lamin-A/C | Nucl | Nuclear assembly | 74.38/6.57 | 18 | 27 |
| 494         | P02545           | LMNA   | Lamin-A/C | Nucl | Nuclear assembly | 74.38/6.57 | 18 | 27 |
| 492         | P02545           | LMNA   | Lamin-A/C | Nucl | Nuclear assembly | 74.38/6.57 | 16 | 25 |
| 483         | P02545           | LMNA   | Lamin-A/C | Nucl | Nuclear assembly | 74.38/6.57 | 15 | 20 |
| 604         | P02545           | LMNA   | Lamin-A/C | Nucl | Nuclear assembly | 74.38/6.57 | 18 | 26 |
| 628         | P14618           | KPYM   | Pyruvate kinase isozymes M1/M2 | Cyt/Nucl | Glycolysis | 58.47/7.96 | 13 | 24 |
| 706         | P00367           | DHE3   | Glutamate dehydrogenase 1, mitochondrial | Mit | Glutamate metabolism | 61.70/7.66 | 11 | 17 |
| 698         | P43490           | NAMPT  | Nicotinamide phosphoribosyltransferase | Cyt | NAD biosynthesis | 55.77/6.69 | 5 | 8 |
| 772         | P06733           | ENOA   | α-Enolase | Cyt | Glycolysis | 47.48/7.01 | 12 | 31 |
| 864         | P04075           | ALDOA  | Fructose-bisphosphate aldolase A | Cyt | Glycolysis | 39.85/8.30 | 6 | 14 |
| 762         | P08670           | VIME   | Vimentin | Cyt | Cytoskeleton component | 53.67/5.06 | 14 | 33 |
| 1354        | P04075           | ALDOA  | Fructose-bisphosphate aldolase A | Cyt | Glycolysis | 39.85/8.30 | 9 | 25 |
| 1355        | P00338           | LDHA   | l-Lactate dehydrogenase A chain | Cyt | Glycolysis | 36.95/8.44 | 11 | 36 |
| 1073        | P17931           | LEG3   | Galectin-3 | Cyt/Nucl/secr | Inflammatory response | 26.19/8.57 | 6 | 18 |
| 1132        | P62826           | RAN    | GTP-binding nuclear protein Ran | Nucl/Cyt | Protein transport | 24.57/7.01 | 6 | 22 |
| 1217        | P04179           | SODM   | Superoxide dismutase [Mn], mitochondrial | Mit | Redox regulation | 24.87/8.35 | 8 | 27 |
| 1215        | P04179           | SODM   | Superoxide dismutase [Mn], mitochondrial | Mit | Redox regulation | 24.87/8.35 | 7 | 24 |
| 1214        | P04179           | SODM   | Superoxide dismutase [Mn], mitochondrial | Mit | Redox regulation | 24.87/8.35 | 7 | 24 |
| 1066        | P40261           | NNMT   | Nicotinamide N-methyltransferase | Cyt | Xenobiotic metabolism | 30.01/5.56 | 6 | 26 |
| 1106        | Q13162           | PRDX4  | Peroxiredoxin-4 | Cyt/secr | Redox regulation | 30.74/5.86 | 7 | 24 |

- Protein spot number according to Fig. 2.
- Protein accession number according to the SwissProt and TrEMBL databases.
- Predicted subcellular localization (Loc) according to PSORT II program. Mit, mitochondria; Cyt, cytoplasmic; Nucl, nuclear; CK, cytoskeleton; secr, secreted, extracellular; ER, endoplasmic reticulum.
- Theoretical molecular weight (M) and pI according to protein sequence and Swiss-2D-PAGE database.
- Number of peptide masses matching the top hit from MS-Fit PMF.
- Amino acid sequence coverage (Cov) for the identified proteins.
- Sequence tag identified by MALDI-TOF/TOF MS/MS.
were collected after 48 h of stimulation. An aliquot of these samples was resolved by SDS-PAGE, along with whole cell extracts. Fig. 5 illustrates the marked differences of proteome and secretome profiles. We then carried out a two-dimensional separation approach for the quantitative study. Proteins from the experimental conditions to be compared (untreated and treated with each brand of CS) were combined 1:1 and resolved by SDS-PAGE. Then the whole gel column was sliced into six sections regardless banding pattern, sections were subjected to in-gel tryptic digestion, and the correspondent peptides were collected for reversed phase nanoLC/MS-MS analysis (19). This procedure resulted in the identification and quantification of 96 proteins present in the culture medium of CS1 condition, 95 proteins present in

| Spot number | Symbol | Protein name | CS1* | CS2* | CS3* |
|-------------|--------|--------------|------|------|------|
| 1           | 257    | GANAB        | -1.5 (+0.4) | -1.3 (+0.2) |
| 2           | 393    | VINC         | -1.2 (+0.1) |
| 3           | 426    | CALD1        | -1.2 (+0.1) |
| 4           | 427    | CALD1        | -1.2 (+0.1) |
| 5           | 447    | CALD1        | 1.2 (+0.1)  |
| 6           | 483    | LMNA         | -1.2 (+0.1) |
| 7           | 490    | LMNA         | -1.2 (+0.1) |
| 8           | 491    | LMNA         | -1.3 (+0.2) |
| 9           | 492    | LMNA         | -1.2 (+0.1) |
| 10          | 494    | LMNA         | 1.2 (+0.1)  |
| 11          | 512    | HSP7C        | 1.2 (+0.1)  |
| 12          | 604    | LMNA         | -1.2 (+0.0) |
| 13          | 698    | NAMPT        | 1.8 (+0.5)  |
| 14          | 706    | DHE3         | -1.2 (+0.1) |
| 15          | 762    | VIME         | 1.3 (+0.0)  |
| 16          | 1066   | NNMT         | 1.2 (+0.0)  |
| 17          | 1073   | LEG3         | -1.2 (+0.1) |
| 18          | 1106   | PRDX4        | -1.3 (+0.1) |
| 19          | 1179   | PSB1         | -1.5 (+0.2) |
| 20          | 1214   | SODM         | 2.8 (+0.4)  |
| 21          | 1215   | SODM         | 2.4 (+0.3)  |
| 22          | 1217   | SODM         | 2.3 (+0.4)  |

* Average volume ratio (± standard error) based on the spots normalized volume across the four groups being compared (each CS treatment and the basal condition, untreated OA chondrocytes) was automatically calculated by SameSpots software. A p value for the one-way analysis of variance <0.05 and a statistical power >0.8 were accepted.
Differential Effects of Chondroitin Sulfate in Chondrocytes

TABLE III
Protein modulations in chondrocytes dependent on CS origin

| Spot number | Symbol | Protein name                                      | CS1/CS2<sup>a</sup> | CS1/CS3<sup>a</sup> |
|-------------|--------|--------------------------------------------------|----------------------|----------------------|
| 1           | 257    | GANAB Neutral α-glucosidase AB                   | -1.6 (+0.4)          | -1.2 (+0.1)          |
| 2           | 393    | VINC Vinculin                                    | -1.2 (+0.1)          | -1.2 (+0.1)          |
| 3           | 426    | CALD1 Caldesmon                                  | -1.2 (+0.0)          | -1.2 (+0.0)          |
| 4           | 447    | CALD1 Caldesmon                                  | -1.2 (+0.0)          | -1.2 (+0.0)          |
| 5           | 483    | LMNA Lamin-A/C                                   | -1.3 (+0.1)          | -1.3 (+0.0)          |
| 6           | 490    | LMNA Lamin-A/C                                   | -1.2 (+0.0)          | -1.3 (+0.0)          |
| 7           | 491    | LMNA Lamin-A/C                                   | -1.2 (+0.0)          | -1.2 (+0.1)          |
| 8           | 492    | LMNA Lamin-A/C                                   | -1.3 (+0.1)          | -1.2 (+0.1)          |
| 9           | 494    | LMNA Lamin-A/C                                   | -1.3 (+0.1)          | -1.2 (+0.1)          |
| 10          | 512    | HSP7C Heat shock cognate 71-kDa protein          | -1.2 (+0.0)          | -1.2 (+0.0)          |
| 11          | 604    | LMNA Lamin-A/C                                   | -1.2 (+0.0)          | -1.2 (+0.0)          |
| 12          | 628    | KPYM Pyruvate kinase isozymes M1/M2              | 1.2 (+0.1)           | -1.2 (+0.0)          |
| 13          | 698    | NAMPT Nicotinamide phosphoribosyltransferase     | 1.9 (+0.5)           | 2.1 (+0.5)           |
| 14          | 762    | VIME Vimentin                                    | -1.3 (+0.0)          | -1.3 (+0.0)          |
| 15          | 864    | ALDOA Fructose-bisphosphate aldolase A           | 1.2 (+0.1)           | -1.3 (+0.0)          |
| 16          | 1066   | NNMT Nicotinamide N-methyltransferase            | 1.3 (+0.2)           | 1.2 (+0.1)           |
| 17          | 1073   | LEG3 Galectin-3                                  | 1.3 (+0.1)           | 1.2 (+0.1)           |
| 18          | 1106   | PRDX4 Peroxiredoxin-4                            | 1.2 (+0.1)           | 1.2 (+0.1)           |
| 19          | 1132   | RAN GTP-binding nuclear protein Ran              | 1.2 (+0.0)           | 1.0 (+0.0)           |
| 20          | 1179   | PSB1 Proteasome subunit β type-1                 | 1.4 (+0.2)           | 1.5 (+0.2)           |
| 21          | 1214   | SODM Superoxide dismutase [Mn], mitochondrial    | 2.9 (+0.5)           | 2.4 (+0.4)           |
| 22          | 1215   | SODM Superoxide dismutase [Mn], mitochondrial    | 2.4 (+0.4)           | 2.2 (+0.4)           |
| 23          | 1217   | SODM Superoxide dismutase [Mn], mitochondrial    | 2.4 (+0.5)           | 2.3 (+0.4)           |
| 24          | 1354   | ALDOA Fructose-bisphosphate aldolase A           | 1.2 (+0.1)           | 1.2 (+0.1)           |
| 25          | 1355   | LDHA l-Lactate dehydrogenase A chain             | 1.2 (+0.1)           | 1.2 (+0.0)           |

<sup>a</sup> Average volume ratio (=standard error) based on the spots normalized volume across the three groups being compared (CS from porcine origin, CS1, and the two CS compounds from bovine origin, CS2 and CS3) was automatically calculated by SameSpots software. A p value for the one-way analysis of variance <0.05 and a statistical power >0.8 were accepted.

TABLE IV
Protein modulations in chondrocytes dependent on CS purity

| Spot number | Symbol | Protein name                                      | CS2/CS3<sup>a</sup> |
|-------------|--------|--------------------------------------------------|----------------------|
| 1           | 257    | GANAB Neutral α-glucosidase AB                   | 1.4 (+0.3)           |
| 2           | 426    | CALD1 Caldesmon                                  | -1.2 (+0.1)          |
| 3           | 427    | CALD1 Caldesmon                                  | -1.2 (+0.1)          |
| 4           | 447    | CALD1 Caldesmon                                  | -1.2 (+0.1)          |
| 5           | 706    | DHE3 Glutamate dehydrogenase 1, mitochondrial    | 1.2 (+0.1)           |
| 6           | 762    | VIME Vimentin                                    | -1.4 (+0.1)          |
| 7           | 772    | ENOA α-Enolase                                   | -1.2 (+0.1)          |
| 8           | 1214   | SODM Superoxide dismutase [Mn], mitochondrial    | -1.2 (+0.1)          |

<sup>a</sup> Average volume ratio (=standard error) based on the spots normalized volume across the two groups being compared (CS2, purity 96.2%, and CS3, purity 99.9%) was automatically calculated by SameSpots software. A p value for the one-way analysis of variance <0.05 and a statistical power >0.8 were accepted.

the culture medium of CS2 condition, and 106 proteins present in the culture medium of CS3 condition. A complete list of all the proteins identified using this strategy is shown in the supplemental Table 2, along with the ProteinPilot information about the confidence of each identification.

A database search was performed to analyze the predicted subcellular localization of the proteins identified by this approach. As expected, most of them were cartilage extracellular matrix proteins or proteins with well-established matrix functions. The most abundant proteins identified in the chondrocyte secretome samples (top four in terms of Protein Pilot hits) included well-known cartilage-related molecules, FN1 (namely fibronectin 1), COL1A2 (collagen α-2(I) chain), CHS1L1 (chitinase-3-like protein 1), and COL12A1 (collagen α-1(XII) chain). We were able to quantify in a relative way most of the identified proteins by comparing their SILAC ratios. 32 of them exhibited a significant modulation of their levels (p < 0.1) because of the pharmacological treatment. These altered proteins are listed in Table V.

The Different CS Compounds Have Various Effects on Chondrocyte Extracellular Proteins—Qualitative and quantitative changes were observed in the secretomes of chondrocytes treated with the different CS compounds. As indicated by the Venn diagram in Fig. 6, each of the studied compounds induces
a characteristic protein profile in OA chondrocytes. CS1 modulated the abundance of 21 proteins: 15 resulted increased, and 6 decreased. CS2 modulated 13 proteins: 5 increased, and 8 decreased. Finally, CS3 modulated 9 proteins: 8 increased, and only 1 decreased. In the case of CS1, more than 60% of the altered proteins were specifically modulated in this condition. The same occurs with CS3, whereas most of the proteins modulated by CS2 are in common with CS1 and/or CS3. Some of the proteins modified by CS1 are known catabolic or inflammatory factors (Fig. 7). We were able to detect interstitial collagenase (MMP1) only in CS1-treated chondrocytes, thus confirming the results obtained by Tat et al. (8). Moreover, MMP3 (stromelysin 1) was identified with the highest protein pilot score in CS1 condition (CS1 score = 17.52; CS2 score = 2.85, and CS3 score = 8.11). Finally, PTX3 (pentraxin-related protein), which plays a role in the regulation of inflammatory reactions, also was significantly increased only by CS1 treatment (ratio CS1/basal = 2.412; p value = 0.0038).

**DISCUSSION**

As a complementary approach to mRNA expression technologies, proteomics is being increasingly applied to drug discovery efforts. The pharmaceutical industry has expressed significant interest in proteomics, with the expectation that this technology will lead to the identification and validation of protein targets and, ultimately, to the discovery and development of viable drug candidates (20). Understanding physiological function, disease, or efficacious and adverse drug effects requires the careful implementation of various proteomics methods (21). In the field of osteoarthritis research, our group exhibits an excellent track record employing two-dimensional electrophoresis for chondrocytes proteomic analysis (11–13, 16, 17, 22, 23). Nevertheless, two-dimensional gels present a number of limitations, such as the difficulties in focusing very acidic proteins (greatly represented in cartilage by the high density and anionic nature of proteoglycans). To avoid these problems and to increase the dynamic range for a more accurate protein quantification (usually, 1–2 orders of magnitude in two-dimensional electrophoresis experiments), we recently developed an alternative method based on SILAC for the quantitative analysis of chondrocytes proteome and secretome (15). The present work illustrates the successful complement of the best quantitative gel-based method up to date (DIGE) for the proteomic analysis of intracellular proteins and the SILAC approach followed by LC-MS analysis for the quantitative study of extracellular proteins. These two proteomic approaches (intracellular and secreted) are largely complementary and exhibited a minimal overlapping: only one protein (vimentin, which is increased by CS3) was identified in both studies. This low correlation demonstrates the utility of combining techniques and analyzing different proteome subsets to enlarge the proteome coverage and increase understanding on the outputs of the drug treatment.

We have employed this dual strategy for evaluating the effect of three different CS compounds on the chondrocytes proteome. CS has shown in some studies to be beneficial for the treatment of OA, but a disparity in its effects has been reported (13). The differences observed could occur depending on the characteristics of the CS employed, including its animal source, purity or structural organization.

**Biochemical Characteristics of CS**—CS is a naturally sulfated glycosaminoglycan formed by the \((1\rightarrow3)\) linkage of \(-\text{D-glucuronic acid to N-acetylgalactosamine; the disaccharide units are associated themselves by } \beta\ (1\rightarrow4)\text{ galactosamine links. The galactosamine residues are sulfated either in position 4 (4-CS) or 6 (6-CS) (24). The sulfation pattern of chondroitin disaccharides and the molecular mass parameters are of paramount importance for chondroitin sulfate properties (25, 26).**

**Supplemental Table 1** shows the characteristics of the different CS employed in this work. CS samples from bovine and porcine origin can be distinguished by the different 4 S/6 S ratios.
CS, like other natural macromolecules, has a complex structure that is known to change with the source tissue, organ, and species (26). Its commercial manufacture relies on bovine, porcine, chicken, or cartilaginous fish (such as shark and skate) by-products, in particular cartilage, as raw material. CS from different sources contains disaccharides possessing sulfate groups in different positions and in different percentages within the polysaccharide chains (27). Moreover, extraction and purification processes may introduce further modifications of the structural characteristics and properties and may lead to extracts more or less rich in chondroitin sulfate, having a variable grade of purity because of the presence of polluting side-products, like other glycosaminoglycans, such as heparin, heparan sulfate, dermatan sulfate, and hyaluronic acid (27). Additionally, chondroitin is administered orally during therapy, and bioavailability and pharmacokinetic parameters have been reported to change depending on its structural characteristics and origin (28, 29). As a consequence, the low quality chondroitin sulfate generally present

![Fig. 6. Modulation by CS of the secretome profile of chondrocytes. As indicated by the Venn diagram, each of the studied compounds induces a characteristic protein profile.](image-url)

### Table V

Extracellular proteins identified by SILAC and LC-MS analysis as differentially altered by CS treatment in OA chondrocytes

| Protein name                          | Symbol | CS1 Ratio<sup>a</sup> Pept<sup>b</sup> EF<sup>c</sup> | CS2 Ratio<sup>a</sup> Pept<sup>b</sup> EF<sup>c</sup> | CS3 Ratio<sup>a</sup> Pept<sup>b</sup> EF<sup>c</sup> |
|---------------------------------------|--------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Actin, cytoplasmic 2                  | ACTG1  | –1.2 10/6 1.1159                                | –1.9 9/4 1.2269                                  | 1.2 10/6 1.064                                  |
| Annexin A2                            | ANXA2  | –1.3 4/2 1.5284                                 | –1.5 6/5 1.4046                                  | –1.3 6/5 1.3617                                 |
| Cartilage oligomeric matrix protein   | COMP   | 1.3 23/11 1.2539                                | 1.5 6/5 1.4046                                  | 1.2 6/5 1.4046                                 |
| Cathepsin D                           | CTSD   | –1.3 4/2 1.5284                                 | –1.5 6/5 1.4046                                  | –1.3 4/2 1.4046                                 |
| Chitinase-3-like protein 2             | CH3L2  | 3.0 3/3 1.7231                                  | 1.3 6/5 1.3617                                  | 1.3 6/5 1.4046                                 |
| Collagen α(1) chain                   | COL1A1 | 1.4 23/14 1.3617                                | 1.2 6/5 1.4046                                  | 1.2 6/5 1.4046                                 |
| Collagen α(1)II chain                 | COL3A1 | 1.6 32/14 1.2838                                | 1.3 6/5 1.4046                                  | 1.3 6/5 1.4046                                 |
| Collagen α(1)III chain                | COL12A1| –1.3 33/21 1.2473                               | –1.2 33/21 1.2473                               | –1.2 33/21 1.2473                               |
| Collagen α(1)IV chain                 | COL15A1| –2.0 1/1 1.2273                                 | –1.6 1/1 1.4171                                 | –1.6 1/1 1.4171                                 |
| Collagen α(3) chain                   | COL6A3 | –2.3 6/6 1.7457                                 | –2.3 6/6 1.7457                                 | –2.3 6/6 1.7457                                 |
| Connective tissue growth factor        | CTGF   | –1.6 1/1 1.3123                                 | –1.6 1/1 1.3123                                 | –1.6 1/1 1.3123                                 |
| Fibronectin                           | FN1    | 1.3 100/43 1.1199                               | 1.3 100/43 1.1199                               | 1.3 100/43 1.1199                               |
| Fibulin-1                             | FBLN1  | 1.5 1/1 1.4159                                  | 1.5 1/1 1.4159                                  | 1.5 1/1 1.4159                                 |
| Follistatin-related protein 1          | FSTL1  | –1.3 4/2 1.5284                                 | –1.3 4/2 1.5284                                 | –1.3 4/2 1.5284                                 |
| Glia-derived nexin                    | SERPINE2| 1.5 4/2 1.6221                                  | 1.5 4/2 1.6221                                  | 1.5 4/2 1.6221                                 |
| Hylauronan and proteoglycan link protein 1 | HPLN1 | –1.5 2/2 1.4204                                | –1.5 2/2 1.4204                                 | –1.5 2/2 1.4204                                 |
| Insulin-like growth factor-binding protein 2 | IGFBP2 | 1.3 7/6 1.2152                                | 1.3 7/6 1.2152                                  | 1.3 7/6 1.2152                                 |
| Insulin-like growth factor-binding protein 3 | IGFBP3 | 1.2 7/6 1.2152                                | 1.2 7/6 1.2152                                  | 1.2 7/6 1.2152                                 |
| Insulin-like growth factor-binding protein 7 | IGFBP7 | 1.4 3/2 1.4892                                | 1.4 3/2 1.4892                                  | 1.4 3/2 1.4892                                 |
| Interstitial collagenase               | MMP1   | 28.7 4/4 1.7749                                 | 28.7 4/4 1.7749                                 | 28.7 4/4 1.7749                                 |
| Metalloproteinase inhibitor 1          | TIMP1  | 1.4 13/6 1.4896                                 | 1.4 13/6 1.4896                                 | 1.4 13/6 1.4896                                 |
| Nucleobindin-1                        | NUCB1  | –1.7 3/3 1.418                                  | –1.7 3/3 1.418                                  | –1.7 3/3 1.418                                 |
| Pentaxin-related protein               | PTX3   | 2.4 9/5 1.6717                                  | 2.4 9/5 1.6717                                  | 2.4 9/5 1.6717                                 |
| Periostin                             | POST   | –1.7 3/3 1.418                                  | –1.7 3/3 1.418                                  | –1.7 3/3 1.418                                 |
| Plasminogen activator inhibitor 1     | SERPINE1| 1.3 2/2 1.2063                                  | 1.3 2/2 1.2063                                  | 1.3 2/2 1.2063                                 |
| Prolargin                             | PRELP  | 1.9 2/2 1.2206                                  | 1.9 2/2 1.2206                                  | 1.9 2/2 1.2206                                 |
| Serpin H1                             | SERPINH1| –1.7 3/3 1.6356                                | –1.7 3/3 1.6356                                 | –1.7 3/3 1.6356                                 |
| Stromelysin-1                         | MMP3   | 13.4 9/5 1.6593                                 | –1.3 21/11 1.2664                               | –1.3 21/11 1.2664                               |
| Tenascin                              | TNC    | 1.2 19/11 1.2271                                | 1.2 19/11 1.2271                                | 1.2 19/11 1.2271                                 |
| Thrombospondin-1                      | THBS1  | 1.8 5/5 1.5978                                  | 1.8 5/5 1.5978                                  | 1.8 5/5 1.5978                                 |
| Transforming growth factor-β-induced protein ig-h3 | TGFBI | –1.3 18/11 1.2444                               | –1.3 18/11 1.2444                               | –1.3 18/11 1.2444                               |
| Vimentin                              | VIME   | 1.5 11/6 1.478                                  | 1.5 11/6 1.478                                  | 1.5 11/6 1.478                                 |

<sup>a</sup> Average SILAC ratios (n = 3) that represent the relative protein abundance in treated (CS1, CS2, and CS3) versus untreated cells, calculated by Protein Pilot 3.0 software.

<sup>b</sup> Number of unique peptides (Pept) used for protein identification and SILAC ratio calculation (both at 95% confidence).

<sup>c</sup> Error factor <\(\sqrt{2}\) (a measure of the error in the average ratio as calculated by Protein Pilot 3.0 software) was accepted for quantification accuracy.
ranging from 12.5 to 2000 μg/ml but generally 200 μg/ml or lower. The relationship between in vitro pharmacological studies and what may be expected in vivo is therefore a matter that has still to be resolved for some target actions. This is particularly evident because, in general, only CS has been investigated for effects in vitro, and virtually no information is available on the mixture of depolymerized or degradation products that are known to exist in inflamed joints (31). Nevertheless, these in vitro investigations (principally using chondrocytes or cartilage explants of bovine or human origin) have provided insight into the likely actions of CS (7, 30, 32–36), including increased synthesis of proteoglycan, hyaluronic acid, and aggregan, blockade of proteoglycan degradation by interleukin-1 and other pro-inflammatory cytokines and metalloproteinases, prevention of oxyrdical formation, modulation of chondrocyte signaling pathways, control of apoptosis, and stress- or aging-related changes in regeneration or repair (36). Thus, it can be summarized that CS has inhibitory effects on multiple cartilage catabolic reactions while also enhancing anabolic processes. Furthermore, several in vivo studies using models of inflammatory joint destruction have confirmed and further established the anti-inflammatory effects of CS (7, 30, 37). Overall, the results of these investigations show that CS fulfills the biochemical requirements for being a biological response modifier at the level of biochemical evidence (31). Although evidence of the effects of degradation products in vitro is still required, these data show that CS itself has defined effects that are unique and influence the degradative processes in OA. All of these considerations underline the interest of the findings reported in this work regarding the in vitro effects of different CS compounds on the chondrocytes proteome and secretome.

CS1 Pro-inflammatory Effect on Chondrocytes Proteome and Secretome—We show how CS1 modulated seven different intracellular proteins compared with basal conditions (Table II). This number rises to 16 when we compared the effect of CS compounds of different purity and origin (CS1 versus CS2 or CS3). Interestingly, as observed in our first pharmacoproteomic study on CS and GS (13), CS seems to affect mainly energy production and metabolic pathways. Proteins related to glycolysis represent the largest functional group increased by CS1 in OA chondrocytes. Furthermore, two other metabolic proteins were also increased: NNMT and nicotinamide phosphoribosyltransferase (NAMT). NAMT, the rate-limiting component in the mammalian NAD biosynthesis pathway, was previously described by our group as increased by IL-1β in chondrocytes (23). Because its expression is upregulated during inflammation, NAMT represents a novel clinical biomarker in rheumatoid arthritis (38). Finally, there is another protein related to glycan metabolism, which is decreased by CS1 and CS3 treatment (Table II): the neutral GANAB. GANAB is an endoplasmic reticulum enzyme that has profound effects on the early events of glycoprotein metabolism and has been recently proposed as biomarker for detecting mild human knee osteoarthritis (27). In this case, our results confirm those obtained previously (13) and demonstrate how GANAB is decreased by CS treatment in normal chondrocytes stimulated with IL-1β and in OA chondrocytes.

Furthermore, we show how CS1 has not only a wide effect on metabolic processes but also seems to regulate cell redox homeostasis. In IL-1β-stimulated chondrocytes, we previously demonstrated that CS combined with GS was able to counteract the IL-1β-mediated increase of SOD2 (13), a known indicator of oxidative stress (28). Inversely, we demonstrate in this study how CS1 increases SOD2 in OA chondrocytes, thus indicating a drug-dependent increase of the presence of superoxide ions in the cell.

Finally, considering the effect of CS1 on the chondrocytes secretome, this compound remarkably increases protein expression levels of MMP1 (ratio CS1/basal = 28.71) and MMP3 (ratio CS1/basal = 13.44). Our results are similar to those obtained by Tat et al. (8) relative to MMP1 (13) but also demonstrate how CS1 significantly reduced anabolic molecules such as collagens (COL12A1 and COL15A1 (collagen-α1 (XV) chain)) and proteoglycans (HPLN1), whereas increased the COMP (cartilage oligomeric matrix protein), a well recognized OA biomarker (39). In addition, CS1 (as described for IL-6) also increased the secretion of another inflammatory mediator: PTX3. Altogether, these results clearly show that CS1 treatment induces a pro-inflammatory state in OA chon-
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chondrocytes (Fig. 7) and underline the need of stricter regulations regarding CS quality control. These should be introduced to guarantee the manufacture of high quality products for nutraceutical utilization and to protect customers from low quality, ineffective, and potentially dangerous products.

**CS2 Effect on Chondrocytes Proteome and Secretome**—In the intracellular analysis, CS2 modulated only two proteins: LEG3 and PSB1 (Fig. 3C and Table II). LEG3 is a molecule involved in acute inflammatory response, which is highly expressed in the synovial tissue of patients with rheumatoid arthritis and represents a novel marker of disease activity in rheumatoid arthritis (40). The decrease of intracellular LEG3 upon CS2 treatment could appear as a beneficial effect of this compound, with this protein being a link molecule between inflammation and joint destruction. On the other hand, PSB1 is a subunit of the proteasome multicatalytic protease complex. As part of the ubiquitin-mediated protein degradation machinery, it is responsible not only for the elimination of misfolded proteins but also for the control of several cellular processes (41). The proteasome takes part in the NF-κB activation process induced by TNF-α and/or IL-1β. NF-κB activation requires the degradation of its inhibitor protein, IκB. In response to cytokine stimulation, the IκBs are phosphorylated by the IκB kinase complex, resulting in the ubiquitination degradation by proteasome and the nuclear translocation of free NF-κB (42). As a result, NF-κB binds to target genes, increasing the expression of many OA-related proteins, such as pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and matrix metalloproteases (MMPs) (33). In chondrocyte cultures, it has been shown that bovine CS diminishes IL-1β-induced NF-κB nuclear translocation (43), although the mechanism for this reduction remains unclear. Our data suggest that the anti-inflammatory activity of CS might be related to its effect on the proteasome. As shown in Table II, both CS2 and CS3 have a similar effect on PSB1, reducing its expression level 1.5- and 1.7-fold, respectively.

The effect of CS2 is more noticeable at the extracellular level, because it modulates 13 different proteins. Most of them are growth factors belonging to the IGF and TGF-β families. IGF-1 is the main anabolic mediator in articular cartilage (2). Its sequestration by high levels of extracellular IGF-binding proteins (IGFBPs) results in a reduced response of chondrocytes. CS2 increased the secretion of three members of this latter family: IGFBP2, IGFBP3, and IGFBP7. On the other hand, TGF-β promotes the synthesis of proteoglycans in cartilage, but it may also induce chondrocyte hypertrophy, osteophyte formation, and fibrosis, which may prevent their application in cartilage repair (2). In CS2 samples, we identified several indications of the down-regulation of the TGF-β pathway, such as the decrease of BGHS (transforming growth factor-β-induced protein ig-h3) and the decrease of tenascin, a matrix protein involved in tissue remodeling, whose expression is known to be induced by TGF-β1 (44).

**CS3 Effect on Chondrocytes Proteome and Secretome**—CS3 modulated eight different intracellular proteins compared with basal conditions: three proteins were increased, whereas five were decreased (Table II). The number of modulated proteins increased (up to 13) when we compared the effect of CS compounds of different purities and origins (CS1 versus CS3) but decreased when we compared the effect of CS compounds of the same origin (CS2 versus CS3) but different purities (Tables III and IV). Interestingly, CS3 seems to affect mainly chondrocyte intracellular and extracellular structural proteins. Both proteomic analyses revealed the increased expression and secretion of vimentin. As recently described, chondrocytes isolated from OA cartilage exhibit a disruption on the vimentin cytoskeleton (45). Therefore, we hypothesize that CS3 could be effective in restoring a proper cytoskeleton network in OA-affected chondrocytes. Among those other modulations caused by CS3, we found an increase of actin and actin-related proteins (actin cytoplasmic 2 and caldesmon), which control cell shape, dynamics, and architecture of chondrocytes. We also found increases in some extracellular matrix components, like PRELP (prolargin), which anchors basement membranes to the underlying connective tissue, and the collagen–α1 (V) chain (46). Finally, among the growth factors, we detected an increase of the connective tissue growth factor (ratio of CS3/basal = 1.59), which promotes chondrocytes proliferation and differentiation (47). Contrary to CS1, these results clearly show that CS3 induces an anabolic response in OA chondrocytes.

**Conclusions**—Proteomic analysis is a useful approach for anti-OA drug screening. Our study provides new information on the effects of different CS compounds on human OA chondrocytes intracellular and extracellular proteomes. Our data indicate that among the three CS compounds tested, CS1 induces the activation of inflammatory and catabolic pathways, whereas CS2 and CS3 induce an anti-inflammatory and anabolic response. This study emphasizes the importance of employing CS compounds of pharmaceutical grade, supported by controlled clinical trials, in the therapy of OA. Based on the diverse effects of CS of different origin and purity in OA chondrocytes, a more strict regulation for quality control should be introduced to guarantee the commercialization of high quality products for pharmaceutical applications or nutraceutical use. Whether the differences in chondrocytes proteome and secretome exposed are translated into a therapeutic effect on the joint tissues remains to be elucidated.

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