

# The Pathobiology of Osteoarthritis and the Rationale for Using the Chondroitin Sulfate for its Treatment

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**Abstract:** Structure-modifying osteoarthritis (OA) drugs are agents that reverse, retard, or stabilize the pathology of OA, thereby providing symptomatic relief in the long-term treatment. The objective of this review is to evaluate the literature on chondroitin sulfate (CS) with respect to the pathobiology of OA to ascertain whether this agent should be classified as a symptomatic slow-acting drugs (SYSADOA), a compound that has a slow onset of action and improve OA symptoms after a couple of weeks.

CS exhibits a wide range of biological activities and from a pharmacological point of view it produces a slow but gradual decrease of the clinical symptoms of OA and these benefits last for a long period after the end of treatment. Many literature data show that CS could have an anti-inflammatory activity and a chondroprotective action by modifying the structure of cartilage. These properties are also related to the oral adsorption of this molecule as high-molecular mass compounds having clusters of sulfate groups and high charge density capable of exert their chondroprotective activity *in vivo*.

**Keywords:** Chondroitin sulfate, Glycosaminoglycans, Oral route, Osteoarthritis.

## THE PATHOBIOLOGY OF OSTEOARTHRITIS

OA and associated musculoskeletal pain and disability are the major causes of morbidity in the United States [1] and other developed nations [2]. Radiological assessment of OA indicates a prevalence in middle-aged individuals of approximately 80%, and this increases markedly with aging producing considerable socioeconomic consequences.

The causes of OA are multifactorial, and although aging is the most strongly associated risk, mechanical, hormonal, and genetic factors all contribute to varying degrees. OA emerges as a clinical syndrome when these determinants result in sufficient joint damage to cause impairment of function and the appearance of symptoms. This clinical syndrome is manifest radiologically by joint space narrowing (due to loss of cartilage) and extensive remodeling of subchondral bone with proliferation at the joint margins (osteophytosis) [3]. In the late stages of disease, these joints are characterized pathologically by extensive cartilage fibrillation, loss of proteoglycans (PGs), and eburnation of bone at sites of high contact stress. Where cartilage is still intact, invasion of the subchondral vasculature into calcified cartilage occurs, accompanied by advancement of the tidemark [4]. Although some chondrocytes may still proliferate and release PGs territorially, many are non-viable. The subchondral bone beneath the regions denuded of cartilage is generally sclerotic and consists of immature woven bone. Histological studies have shown that the marrow spaces of the cancellous bone, particularly in the hip

joint, may become engorged with lipid, cholesterol, and fibrin deposits [5].

Products of cartilage breakdown have been shown to be antigenic [6], and when released into synovial fluid due to excessive catabolism, may provoke synovitis. This synovial inflammation, once established, can alter the metabolism of resident synoviocytes, the major biosynthetic source of hyaluronan (HA) in synovial fluid [7]. Inflammatory mediators released from local synovial cells and infiltrating leukocytes can promote increased vascular permeability and the accumulation of plasma in synovial fluid, thereby decreasing HA concentration. This dilution of HA and reduction in its molecular weight due to abnormal synthesis by synoviocytes results in a decrease in the viscoelasticity of synovial fluid and thus its ability to lubricate and protect articular cartilage. Macrophages of the synovium and the leukocytes that have entered the synovial cavity are also abundant source of cytokines, procoagulant factors, proteases, oxygen-derived free radicals and nitric oxide.

Although much of the excess proteolytic activity released into synovial fluid is abrogated by the endogenous inhibitors present, cytokines and free radicals can freely diffuse into cartilage and downregulate PG and collagen synthesis by chondrocytes. These cytokines can also initiate the production of catabolic proteases, cytokines, and nitric oxide by the cartilage cells, which contribute to further matrix destruction through paracrine pathways [8].

## PHARMACOLOGICAL TREATMENT OF OA

Pharmacological management in OA has targeted the symptoms of the disease rather than the underlying cause. Analgesics, steroidal, and nonsteroidal antiinflammatory

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drugs (NSAIDs) represent the mainstay of treatment [9, 10]. However, the deleterious side effects associated with the use of many of these agents has led to a more conservative approach to their use in recent years.

The discovery of two isoforms of cyclooxygenase (COX), the enzymes encoded by different genes and responsible for the conversion of arachidonic acid into prostaglandin E<sub>2</sub>, has provided new impetus for the use of NSAIDs in the management of OA. In the gastrointestinal tract, a constitutive form of cyclooxygenase, COX-1, is mainly responsible for the synthesis of eicosanoids, including PGE<sub>2</sub>. The other form of cyclooxygenase, COX-2, is rapidly synthesized when the cell is stimulated by exposure lipopolysaccharides or proinflammatory cytokines [11, 12]. COX-2 is thus an inducible enzyme, which is produced by cells in response to activation by cytokines or bacterial invasion. Aspirin, indomethacin, naproxen, piroxicam and ibuprofen are predominantly inhibitors of COX-1, while diclofenac is approximately equipotent against COX-1 and COX-2. Meloxicam, aceclofenac and flosulide, in contrast, are reported to be selective inhibitors of COX-2 at low plasma concentrations [13, 14].

## THE RATIONALE FOR THE USE OF CS IN OA

CS is an important component of most vertebrate tissues predominantly present in the extracellular matrix. It is most abundant in the connective tissues of the body, cartilage, skin, blood vessels and also bone, ligaments and tendons. These tissues also contain large amounts of collagen. Where the collagen has a single predominant orientation, such as in ligaments and tendons, the tissues are tensile and the CS content is quite low, but where there is no predominant orientation of the collagen, such as in skin, there is a high content of CS and the tissue stretches in tension, but is resilient and resists compression.

CS is a polyanion (see below) and some of its properties are due to its strong charge capable to draw water into tissues and hydrates them. CS chains are synthesized by cells covalently attached to proteins, which are secreted into the extracellular matrix as PGs. Several families of PGs bearing CS chains have been characterized, including two of the most prominent families of PGs found in the extracellular matrix of connective tissues, the aggrecan family and the leucine-rich repeat family [15, 16]. The aggrecan family are high molecular weight (> 500 kDa) and aggregate extracellularly by binding to HA. The leucine-rich

repeat family are of smaller size (< 200 kDa) and contain two members, decorin and biglycan, that contain CS/dermatan sulfate chains.

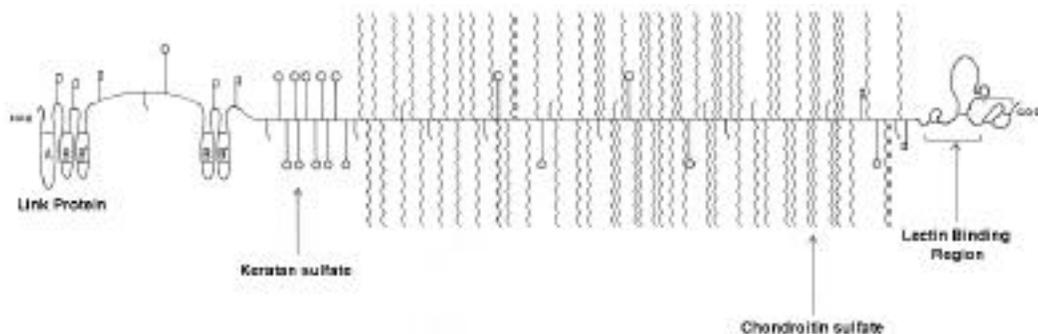
Articular cartilage is a very specialized tissue with a large expanded extracellular matrix composed of more than 98% of its volume matrix and less than 2% cells. The properties of this tissue are related to the contribution made by fibrillar collagen and nonfibrillar PGs. The structure of collagen gives it impressive tensile properties and this is utilized in cartilage in a special way to produce a tissue that is not only strong in tension but also resistant to compression by filling the interfibrillar matrix with a very high content of CS-rich PG, primarily aggrecan. The aggrecan at high concentration draws water into the tissue, which swells and expands the matrix placing the collagen network under tension. At this equilibrium, with the tissue swollen with water, it has good compressive resilience.

In degenerative joint diseases there is loss of the articular cartilage. A key point in the degenerative process is the loss of PG from the cartilage and the exposure of its collagen network to mechanical disruption. However, the results from studies of experimental osteoarthritis suggest that early in the process there is a hypermetabolic repair response in articular cartilage with increased synthesis of matrix components and increased matrix turnover [17]. The increased synthesis of aggrecan in cartilage is accompanied by interesting changes in the CS, which shows a longer chain length and the chains contain more epitopes recognized by specific antibodies [18]. Although CS chains are typically very poorly antigenic, monoclonal antibodies that recognize some specific sequences within the chains were produced. These CS clusters are infrequently found in normal healthy tissue, but their expression increases in cartilage during the early hypermetabolic response in experimental OA.

The biological reason for these changes in CS structure in cartilage in early OA is not well clear, but it may suggest that there are specific biological functions of selected sequences within CS chains that may be important to the processes of tissue repair.

## STRUCTURE AND FUNCTION OF PG

As previously reported, CS is a key molecule in the composition of PG. As a consequence major details concerning the structure and function of these



**Fig. (1).** Schematic representation of the structure of the aggrecan showing the core protein and the polysaccharides.

macromolecules will be discussed.

### Major PG of Cartilage

The predominant proteoglycan in cartilage is a large macromolecule with extreme polydispersity in size and composition, named aggrecan (Fig. 1). A typical molecule has an Mr of approx. 2,500 kDa, consisting of a core protein (estimated Mr = 200-300 kDa) with about 100 CS chains and up to 130 keratan sulfate chains. The keratan sulfate chains are bound to the protein by linkage structures closely related to the numerous  $\alpha$ -linked oligosaccharides, which are also found distributed along the core protein. Other substituents on the protein include about 10-15 N-linked oligosaccharides of the complex type, and phosphate esters on up to 80% of the xylose residues that initiate CS chains as well as a few on serine residues.

The properties of the glycosaminoglycan chains, which account for 80-90% of the mass, dominate the properties of the PG. In some cases, variation in glycosaminoglycan chain type, number, and length can account for all the size polydispersity of newly synthesized PGs. In other cases, these parameters account for some, but not all, of the size polydispersity present in the PG population. For example, some studies of PGs isolated from hyaline cartilages indicate that variations occur in the size of the core protein. This variation may be continuous, giving rise to polydispersity, or discontinuous, giving rise to heterogeneity.

The distribution of substituents along the core protein reflects regional specialization of the polypeptide. A portion at the N-terminus with Mr = 60-70 kDa, referred to as the hyaluronic acid-binding region, contains:

1. few or no glycosaminoglycan chains,
2. most of the N-linked oligosaccharides,
3. a binding site that interacts specifically with hyaluronic acid to permit the formation of large aggregates, and
4. a site that interacts with the link proteins involved in aggregate stabilization.

An adjacent region of the core protein, Mr = approximately 30 kDa and referred to as the keratan sulfate-attachment region, contains 60% or more of the keratan sulfate chains, while the remainder towards the carboxyl terminus, referred to as the CS-attachment region, contains the majority of the CS chains as well as a few of the keratan sulfate chains and  $\alpha$ -linked oligosaccharides (Fig. 1).

The ability of the core protein of this PG to interact with hyaluronic acid distinguishes it from other core protein types, more so than does its size or composition. PG binds to hyaluronic acid with highly specific interactions and the link protein stabilizes this interaction. Neither the N-linked oligosaccharides on the PG nor those on the link protein appear to be critically involved in the aggregation process. Biosynthetic studies have established that aggregation occurs extracellularly. Recent developments using hyaline cartilage organ cultures indicate that newly synthesized PGs cannot bind to hyaluronic acid as well as the same molecules do after being allowed to "mature" in the tissue for a period of

time, typically 24 hours. These results suggest that different conformational states of the hyaluronic acid-binding region with different affinity for hyaluronic acid may exist.

### Small PGs in Collagenous Connective Tissues

Most, perhaps all, fibrillar collagenous connective tissues contain one or more small PGs, usually with dermatan sulfate/CS as the glycosaminoglycan constituent. Members of this group typically have core proteins with estimated Mr = 40-50 kDa, with one or two glycosaminoglycan chains attached. N-linked oligosaccharides, and in some cases,  $\alpha$ -linked oligosaccharides are also present on the core protein. Recent work with these PGs isolated from cartilage indicates that two distinctly different core proteins are probably present in this group. Additionally, a unique small PG with keratan sulfate chains has been isolated from cornea. Some features of these PGs in different connective tissues are described in the following sections.

#### Cartilage

A low-buoyant-density, small PG has been isolated from bovine nasal cartilage and chemically characterized. Chondroitinase digestion of the macromolecule produced a core preparation of Mr = 45 kDa when analyzed by SDS-PAGE. It contained one to two CS chains of Mr = approximately 35 kDa with no evidence for the presence of dermatan sulfate. The core protein was unusually rich in leucine. This PG had been observed in several cartilages from baboon, in bovine articular cartilage, in chick growth plate cartilage, and as a biosynthetic product in organ culture of bovine articular cartilage.

A similar population of PGs has been isolated from mature bovine articular cartilage, which contains about 10 times more of these PGs than does bovine fetal epiphyseal cartilage. In dissociative solvents, only one size class of PG was observed with Mr of 90-120 kDa. Additionally, after chondroitinase digestion, a core preparation with closely spaced bands near Mr = 45 kDa on SDS-PAGE was obtained. The glycosaminoglycan chains contained significant amounts of iduronic acid and are therefore classified as dermatan sulfate. A subpopulation of the PGs, referred to as dermatan sulfate PG-I, exhibited an unusual self-aggregation, migrating as a broad band with apparent Mr = 165-285 kDa when electrophoresed on SDS-PAGE gels at high ionic strength; whereas a second subpopulation, referred to as dermatan sulfate PG-II, electrophoresed in the expected Mr = 90-120 kDa range. Further analyses revealed that PG-I and PG-II have distinctly different antigenic properties; polyclonal antibodies against the mixed population detected only PG-II. After chondroitinase digestion of PG-II, followed by SDS-PAGE and immunotransfer, the antiserum stained two closely spaced bands at Mr = 43 and 47 kDa, similar to results observed for this family of PGs isolated from a number of tissues. Amino acid sequence analyses indicate that PG-II has an identical N-terminal sequence as that for the small PG from skin whereas PG-I does not have the same N-terminal sequence. Neither PG-I nor PG-II cross-reacts with antisera against the larger, aggregating PG species from cartilage. The mechanism for the self-association of PG-I is not clear, although it may be mediated in part by the self-association of the dermatan sulfate chains.

There were no clear differences in the chemical properties of the dermatan sulfate chains for PG-I and PG-II.

### **Tendon**

Investigations of the small PGs from tendon provide several lines of evidence that suggest that these PGs are closely associated with collagen fibrils. Almost 90% of the PGs isolated from proximal bovine flexor tendon, which is under essentially only tensile load, are in the small PG family. As with the small PGs from cartilage described above, these tendon PGs have a small proportion of PG-I molecules as indicated by their ability to self-associate, although PG-II predominates. The glycosaminoglycan chains are dermatan sulfate, with approximately 70% iduronic acid and with average Mr = 37 kDa. After chondroitinase digestion, a core preparation with an apparent Mr = 48 kDa was produced. The intact PG as well as to a lesser extent, the core preparation, can inhibit fibrillogenesis of type I and type II collagen in an *in vitro* assay system, whereas the isolated dermatan sulfate chains have no effect. Interestingly, the population of small PGs isolated from either cartilage or aorta has no effect on fibrillogenesis. The results suggest that the core protein of the small dermatan sulfate PG from tendon can bind selectively to collagen and inhibit fibrillogenesis, and differs in this respect from other PGs in this family. The small PG from tendon has the same N-terminal sequence as that of the small PG from skin. An antiserum to these PGs recognizes cartilage PG-II, but not PG-I, and cross-reacts with bovine bone PG-II.

Electron microscopic studies of developing and mature rat tendon utilizing cuprinolinic blue as a selective stain for PG showed that the PGs are periodically arrayed on the collagen fibril. Further, there was a correlation between the dermatan sulfate content of the tendon and the average diameter of the collagen fibrils in tendons from animals of different age. Because the predominant PGs in this tissue are the small species, these results suggest that they have a function in either tissue collagen morphogenesis, or tissue maintenance, or both. The periodic association of PG with collagen has been seen in other tissues, notably aorta where small matrix granules align with the collagen fibril.

### **Bone**

A sequential extraction procedure, 4 M guanidine HCl followed by 4 M guanidine HCl with 0.5 M EDTA, was used to isolate a PG population in the second extract that was present in the mineral phase of fetal bovine bone. The predominant PGs in this population were in the small PG family and contained CS chains (no dermatan sulfate was found) of Mr = 40 kDa. On SDS-PAGE analyses, two broad PG bands were seen; the smaller (equivalent to cartilage PG-II) was suggested to have a single CS chain, while the larger (equivalent to cartilage PG-I) was suggested to be the same core protein with two glycosaminoglycan chains. Chondroitinase digestion of the mixed PG population yielded core preparations with the characteristic two-band pattern around Mr = approximately 43 kDa. More refined analyses since then have shown that the core preparation derived from bone PG-I appears to give a single band at Mr = 45 kDa, while that from bone PG-II gives the doublet at Mr = 43 and 46 kDa. PG-I and PG-II have significantly different amino acid compositions. PG-I had a composition

very similar to that of the low-buoyant-density PG isolated from bovine nasal cartilage, characterized by an unusually high leucine content. PG-II had lower leucine and higher amounts of aspartate/asparagine and glutamate/glutamine. Bone PG-I does not have dermatan sulfate, or has dermatan sulfate with low iduronic acid content. Antisera raised against bone PG-II cross-react with tendon and cartilage PG-II.

### **Skin**

Several investigators have reported that skin fibroblasts synthesize both small and large PGs. The large PGs have core preparations after chondroitinase with Mr = approximately 500 kDa, and the small, predominant PGs appear to be primarily PG-II. Recently, radiolabeled precursors and antisera against the core protein of the small PG population were used to study their biosynthesis in the presence or absence of tunicamycin, which inhibits N-linked oligosaccharide synthesis. The results suggested that the characteristic doublet observed for core preparations at Mr = 43 and 47 kDa result from the presence of different numbers, either two or three, of N-linked oligosaccharides on the core protein. They also suggested that this PG did not contain any  $\alpha$ -linked oligosaccharides and that the core protein devoid of any N-linked oligosaccharides has a Mr = 39 kDa.

A small dermatan sulfate PG isolated from porcine skin, has similar overall properties to those described above. This PG population was also isolated from bovine skin and has been sequenced through the first 24 amino acids from the N-terminus. Position 4 of the sequence contains a serine residue substituted with a complex carbohydrate, quite likely a glycosaminoglycan chain. Interestingly, the core protein of a small PG isolated from human placental membrane has an identical amino acid sequence through the first nine residues, after which it differs.

### **Cornea**

Intact PGs have been isolated from bovine and monkey cornea by dissociative extraction and ion exchange chromatography. Two major PG populations of small molecular size were identified, one with dermatan sulfate chains and one with keratan sulfate chains. The hydrodynamic size distribution of these PGs permit them to fit well within the space between the regular lattice of collagen fibrils in the corneal stroma, and it is presumed that the PGs facilitate and maintain the orderly packing of the collagen fibrils required for the optical transparency of this tissue. In support of this, disorganization of the collagen fibril packing is observed during corneal wound healing where distinctly different and larger PGs are synthesized; and the human disease corneal macular dystrophy, which also involves disorganization of the matrix, is characterized by altered synthesis of the keratan sulfate PG. The dermatan sulfate PG population is related to the PG-II PGs discussed above as indicated by the production of the characteristic core preparation doublet at Mr = 43 and 47 kDa after chondroitinase digestion, and by having similar immunological properties, amino acid composition, and peptide maps as the small PG populations from bone, tendon, and sclera. These corneal PGs contain one to two dermatan sulfate chains of Mr = 55 kDa, distinguished by

their low level of sulfation, as well as N-linked oligosaccharides.

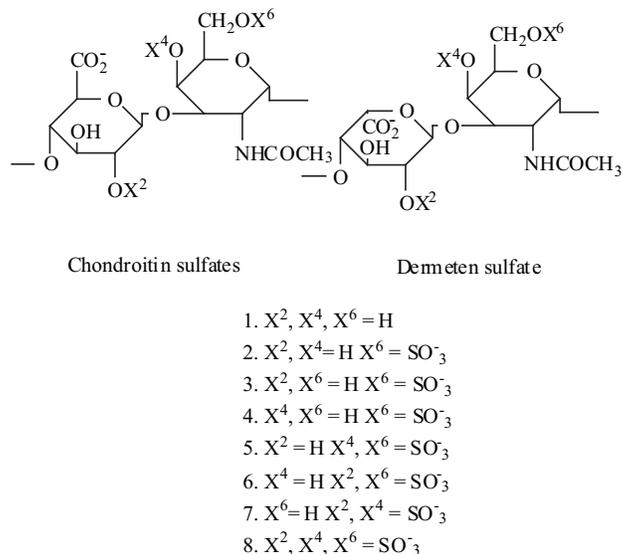
The keratan sulfate PG is considered unique to the cornea. Two studies have used keratanase digestion of bovine corneal PGs followed by SDS-PAGE to estimate the size of the core preparation for the keratan sulfate PGs. In one [19], in which the PGs were purified by ethanol precipitation and step elution from an ion exchange column, two bands with apparent Mr = 40 and 55 kDa, were observed. In the other, in which the PGs were isolated by gradient elution from an ion exchange column, a major band with apparent Mr = 53 kDa was observed with only a faint band at 40 kDa. It remains to be determined whether the smaller core protein band is derived from the larger, possibly as a degradation product. The PG contains two to three branched oligosaccharide linkage regions with a structure related to complex N-linked oligosaccharides; each contains two keratan sulfate chains. High-mannose N-linked oligosaccharides are also present, one of which has an unusual four-mannose residue structure, and which may be related to synthesis of the keratan sulfate linkage oligosaccharide region. Antibodies against the core protein of the keratan sulfate PGs from bovine and monkey cornea do not cross-react with the corneal dermatan sulfate PG population. The difference in size of the core protein as well as in its immunological determinants makes it likely that the core protein of the keratan sulfate PG is a separate gene product.

## CS STRUCTURE

Due to the presence of CS as a fundamental component of various PGs above described, its structure is strictly related to the kind of cells, tissues, organs and species. Generally, CS is an unbranched, polydisperse, complex glycosaminoglycan extracted and purified from various tissues, with polysaccharide chains composed mainly of disaccharide units of sequence [-N-acetyl-D-galactosamine-( $\beta$  1:4)-D-glucuronate-( $\beta$  1:3)-]. Depending on the source, different non-sulfated and sulfated disaccharides are present within the polysaccharide chains [20, 21]. Due to the presence of sulfate groups in different amounts and located in various positions (2 and 3 of uronic acid, and 4 and 6 of N-acetyl-galactosamine residues), CS represents a very heterogeneous family of polysaccharides, in terms of degree of sulfatation, molecular mass, relative amounts of iduronic acid and glucuronate, depending on the tissue of origin.

The regular disaccharide sequence of CS A, chondroitin-4-sulfate, is constituted by [(1 $\rightarrow$ 4) - O - (D-glucopyranosyluronic acid) - (1 $\rightarrow$ 3) - O - (2-N-acetamido-2-deoxy-D-galactopyranosyl-4-sulfate)]. CS C, chondroitin-6-sulfate, is mainly composed of a disaccharide unit [(1 $\rightarrow$ 4)-O-(D-glucopyranosyluronic acid)-(1 $\rightarrow$ 3)-O-(2-N-acetamido-2-deoxy-D-galactopyranosyl-6-sulfate)] (Fig. 2). Polysaccharide chains of dermatan sulfate (CS B) consist of a prevailing disaccharide unit [(1 $\rightarrow$ 4)-O-(idopyranosyluronic acid)-(1 $\rightarrow$ 3)-O-(2-acetamido-2-deoxy-D-galactopyranosyl-4-sulfate)]. Disaccharides with different number and position of sulfate groups can be located, in different percentages, inside the polysaccharide chains, such as the non-sulfated or disulfated disaccharide in which two sulfate groups are O-

linked in position 2 of D-glucuronic acid and 6 of N-acetyl-D-galactosamine (disaccharide D) or in position 4 and 6 of N-acetyl-D-galactosamine (disaccharide E) (Fig. 2). These heterogeneous structures are responsible for different and more specialized functions of these glycosaminoglycans.



**Fig. (2).** Structures of disaccharides forming chondroitin sulfate species.

## BIOLOGICAL PROPERTIES OF CS

CS exhibits a wide variety of biological functions mainly due to the presence of rare oversulfated structural building units that form domain structures that interact specifically with other molecules, such as the regulation of neuronal patterning in the retina [22], interactions with fibronectin [23], neurite outgrowth promoting activity [24], modulation of the adhesive function of  $\alpha_4\beta_1$  integrin [25], activation of monocyte and B-cell [26], and activation of plasminogen [27].

Highly sulfated PG are correlated with axon boundaries in the developing central nervous system, which suggests that these molecules affect neural pattern formation. In the developing mammalian retina, gradual regression of CS may help control the onset of ganglion cell differentiation and initial direction of their axons. Changes induced by the removal of CS from intact retinas in culture confirm the function of CS in retinal histogenesis [22].

Interactions between fibronectin (FN) and glycosaminoglycans are essential for extracellular matrix morphology and cell adhesion. Recombinant FNs (deminectins (DN)) containing the carboxyl-terminal cell, heparin, and fibrin domains bind specifically to CS in affinity chromatography assays [23]. Using a panel of mutant DNs, important determinants for CS binding have been localized to repeats III13 and III14 within the heparin domain. In particular, mutation of an arginine pair in repeat III13 to neutral residues ablated binding to chondroitin sulfate as previously reported for heparin [28]. These results, in combination with the ability of heparin and CS to compete for binding to DNs, demonstrate that these two glycosaminoglycans interact with similar or overlapping

sites in FN. One important difference between FN interactions with heparin and CS is that, while FN and DNs bound equally to heparin, FN bound less efficiently than DNs to CS. Reduced binding to CS was also observed with a larger recombinant FN lacking internal repeats III1-7 indicating that the amino-terminal region acts to limit binding to the carboxyl-terminal domain. These results demonstrate that interactions between FN and CS are modulated by molecular context.

A mouse brain CS-PG, DSD-1-PG, bears the DSD-1 epitope and has neurite outgrowth promoting properties. Shark cartilage CS-C inhibits the interactions between the DSD-1-specific monoclonal antibody 473HD and the CS chains of the DSD-1-PG, which is expressed on the mouse glial cells [29]. On the other hand, several hexasaccharides isolated from commercial shark cartilage CS-D, which contains a higher proportion of characteristic D units (GlcUA(2-sulfate)beta1-3GalNAc(6-sulfate)) as compared with CS-C, has the A-D tetrasaccharide sequence composed of an A disaccharide unit (GlcUAbeta1-3GalNAc(4-sulfate)) and a D disaccharide unit [30]. CS-D inhibited the interactions between monoclonal antibody 473HD and DSD-1-PG and also promoted neurite outgrowth of embryonic day 18 hippocampal neurons. Eight octasaccharide fractions were isolated from CS-D after partial digestion with bacterial chondroitinase ABC by means of gel filtration chromatography and anion-exchange high performance liquid chromatography to investigate the frequency and the arrangement of the A-D tetrasaccharide unit in the polymer sequence. Structural analysis performed by a combination of enzymatic digestions with 500-MHz  $^1\text{H-NMR}$  spectroscopy demonstrated that the isolated octasaccharides shared the common core structure DeltaHexAalpha1-3GalNAcbeta1-4(GlcUAbeta1-3GalNAc)3 with four, five, and six sulfate esters at various hydroxyl groups in different combinations. In the structure, DeltaHexA and GlcUA represent 4-deoxy-alpha-L-threo-hex-4-enopyranosyluronic acid and glucuronic acid, respectively. No D-D tetrasaccharide sequence was found, and discrete D disaccharide units were demonstrated exclusively as A-D tetrasaccharide units in either an A-D-A or an A-D-C hexasaccharide sequence in the five octasaccharides that represented about 5.0% (w/w) of the starting polysaccharides (C denotes the disaccharide GlcUAbeta1-3GalNAc(6-sulfate)). It remains to be determined whether such characteristic hexasaccharide sequences present in shark cartilage CS-D serve as functional domain structures recognized by some protein ligands.

The alpha4beta1 (but not alpha5beta1) integrin-mediated melanoma cell adhesion is inhibited by removal of cell surface CS glycosaminoglycan (CSGAG), suggesting that melanoma CS proteoglycan plays a role in modulating the adhesive function of alpha4beta1 integrin. Alpha4beta1 integrin binds to CSGAG. A peptide from within alpha4 integrin termed SG1 (KKEKDIMKKTI) that binds to cell surface melanoma CS-PG, indicating that SG1 represents a CSGAG binding site within the alpha4 integrin subunit, was identified. Soluble SG1 inhibits alpha4beta1 integrin-mediated human melanoma cell adhesion to CS1. Polyclonal antibody generated against the peptide inhibits melanoma cell adhesion to CS1, and the inhibition is reversed by  $\text{Mn}^{2+}$  and an activating monoclonal antibody anti-beta1 (8A2). Additionally, pretreatment of cells with

anti-SG1 IgG inhibits the expression of the monoclonal antibody 15/7 epitope in the presence of soluble CS1 peptide, suggesting that anti-SG1 IgG prevents ligand binding by alpha4beta1 integrin. These results demonstrate that alpha4beta1 integrin interacts directly with CSGAG through SG1 site, and that this site can affect the ligand binding properties of the integrin [25].

At inflammatory sites, PG are both secreted by activated mononuclear leukocytes and released as a consequence of extracellular matrix degradation. Chondroitin 4-sulfate PGs constitute the predominant ones produced by activated human monocytes/macrophages. Two chondroitin 4-sulfate forms, CSA and CSB, can activate distinct peripheral blood mononuclear cell types [26]. Whereas CSA activates monocytes (to secrete monokines), CSB activates B-cells (to proliferate). In contrast, the chondroitin 6-sulfate CSC and heparin do not exert these functional effects. It was further showed that CD44 monoclonal antibodies block CSB-induced B-cell proliferation. These findings point to glycosaminoglycans, and specifically chondroitin 4-sulfates, as a novel class of immunological mediators at inflammatory sites. Furthermore, the data link CD44 to B-cell activation, paralleling the established roles of CD44 in T-cell and monocyte activation.

CS E markedly enhanced plasminogen activation by tissue plasminogen activators (t-PAs) and urinary plasminogen activator (u-PA) *in vitro*; in the presence of 10 microg/ml of CS E, the potentiation factors of single chain of t-PA, two chain of t-PA and u-PA were 400, 140 and 130, respectively [27]. Though the potentiation activity of CS E gradually decreased when it was depolymerized by chondroitinase ABC, the specific disaccharide from CS E still showed significant activity. Glycosaminoglycan from sea cucumber, which possesses a very similar core structure to CS E, but has additional sulfated fucose branches exhibit very weak activity. These results suggested that the minimal structural requirement in CS E to enhance plasminogen activation by plasminogen activators is GlcUAbeta1-3GalNAc(4S,6S) and that additional branching sugars abolish the activity.

## ANTI-INFLAMMATORY EFFECT OF CS

CS has anti-inflammatory activity and affects cartilage metabolism. Oral administration of CS was shown to significantly decrease the granuloma formation because of cotton, pellet or sponge implants, the inflammatory response in adjuvant arthritis, and lysosomal enzyme release in carrageenan pleurisy. Furthermore, CS and its fractions inhibited the directional chemotaxis induced by zymosan-activated serum, are able to decrease the phagocytosis and the release of lysozyme induced by zymosan and to protect the plasma membrane from oxygen reactive species. Compared with nonsteroidal anti-inflammatory drugs (indomethacin, ibuprofen), CS appears to be more effective on cellular events of inflammation than on edema formation [31]. When the effects of CS on chymopapain-induced articular cartilage injury in rabbits were investigated, it was found that rabbits that were given intramuscular or oral doses of CS before injection of chymopapain demonstrated less PG loss from the articular cartilage than the controls

[32]. In an *in vitro* study, Bassleer and Malaise found that the addition of CS to the culture medium resulted in an increase in PG concentration of the pericellular matrix and a dose-dependent decrease in collagenolytic activity released from human articular cartilage of chondrocytes in culture [33].

## CLINICAL STUDIES

Mazieres *et al.* treated 120 patients with CS in a placebo-controlled study. Joint pain, as measured on a visual analog scale, the Lequesne index, and patient and physician global assessments all showed significant improvement in the CS-treated group [34]. One hundred twenty patients with osteoarthritis of the knees and hips were entered into a randomized, placebo-controlled, double-blind trial designed to evaluate the effectiveness of CS. The three-month treatment phase was followed by a two-month treatment-free phase to allow evaluation of carry-over effects. The main endpoint was use of nonsteroidal antiinflammatory drugs (expressed as mg of diclofenac equivalent). At completion of the three-month treatment phase, patients taking CS (4 capsules/day) were using significantly less NSAIDs; this decrease persisted throughout the two-month treatment-free follow-up phase. The other parameters studied including visual analog scale assessment of pain, the Lequesne pain-function index, and overall patient and physician assessments, all showed a similar significant tendency. Tolerance was outstanding and no patients required premature withdrawal. These findings indicate that CS is useful for the treatment of osteoarthritis, both as an agent slowly effective against symptoms and to reduce the need for NSAIDs. The carry-over effect of the drug suggests that intermittent administration may be appropriate.

56 patients with knee OA were enrolled in a 1-year double-blind placebo controlled study comparing 800 mg of CS with placebo; forty-seven patients (25 CS-treated, 22 placebo) completed 1 year [34]. Mobility, joint effusion and swelling were significantly better in the CS group.

In a study by Bucsi and Poor, 40 patients treated with 800 mg of CS were compared with 40 who received placebo. The CS group showed significant improvement in the Lequesne index, visual analog pain scale, 20-meter walk time, and patient and physician efficacy ratings. The CS group used significantly less paracetamol than the placebo group [35]. Patients with OA of the knee were treated with CS (Chondrosulf, IBSA, Lugano, CH) in a randomized, double-blind, placebo-controlled study, performed in two centres. The efficacy and tolerability of oral CS capsules 2 x 400 mg/day vs placebo was assessed in a 6-month study period. Patients with idiopathic or clinically symptomatic knee OA, with Kellgren and Lawrence radiological scores I-III, were included in this trial. Clinical controls were performed at months 0, 1, 3 and 6. Eighty patients completed the 6-month treatment period. Lequesne's Index and spontaneous joint pain (VAS) decreased constantly in the CS group; on the contrary, slight variations of the scores were reported in the placebo group. The walking time, defined as the minimum time to perform a 20-meter walk, showed a statistically significant constant reduction only in the CS group. ANOVA with repeated measures showed a

statistically significant difference in favor of the CS group for these three parameters. During the study, patients belonging to the placebo group reported a higher paracetamol consumption, but this consumption was not statistically different between the two treatment groups. Efficacy judgements were significant in favor of the CS group. Both treatments were very well tolerated. All these results strongly suggest that chondroitin sulfate acts as a symptomatic slow-acting drug in knee OA.

Bourgeois *et al.* evaluated 40 patients with knee OA who were treated with CS as a 1200 mg dose oral gel. Forty-three patients were treated with 400 mg capsules three times per day, and 44 received placebo. The Lequesne index and pain score (visual analog scale) decreased significantly in both CS groups, compared with the placebo group, and patient and physician global assessments significantly favored the CS-treated group [36]. This multicenter randomized, double-blind, controlled study was performed to compare the efficacy and tolerability of CS (Condrosulf, IBSA, Lugano, CH) 1200 mg/day oral gel vs CS 3 x 400 mg/day capsules vs placebo, in patients with mono or bilateral knee osteoarthritis (Kellgren and Lawrence radiographic score grade I to III). A total of 127 patients, 40 of whom were treated with CS 1200 mg/day, 43 with CS 3 x 400 mg/day and 44 with placebo, were included in the statistical analysis of this 3-month treatment study. In the CS groups, Lequesne's Index and spontaneous joint pain (VAS) showed a significant reduction of clinical symptoms ( $P < 0.01$  for both parameters), while only a slight reduction was observed in the placebo group ( $P = ns$  for Lequesne's Index and  $P < 0.05$  for VAS). The physician's and patient's overall efficacy assessments were significantly in favour of the CS groups ( $P < 0.01$ ). The treatment carried out with the three formulations was very well tolerated. In conclusion, these results indicate that CS favors the improvement of the subjective symptoms, improving the joint mobility. An additional consideration is that the efficacy of 1200 mg CS as a single daily dose does not differ from that of 3 x 400 mg daily doses of CS for all the clinical parameters taken into consideration.

Morreale and co-workers [37] evaluated 146 patients with knee OA. Seventy four patients received 400 mg CS three times per day for 3 months, followed by 3 months without treatment. These patients were compared with 72 patients who were treated with 50 mg of diclofenac three times a day for 1 month, followed by 5 months of treatment. Those treated with diclofenac had a decrease in joint pain at 10 days, which disappeared shortly after discontinuation of treatment; those treated with CS had a significant response at 30 days, which lasted for 3 months after discontinuation of the drug. The Lequesne index after 3 months of CS treatment was 78% lower than at baseline; after 30 days of diclofenac it was 62.6% lower. Three months after discontinuation of CS, the Lequesne index remained 64.4% lower than the pre-treatment level. Three months after discontinuation of diclofenac the index was only 29.7% lower than at baseline. Thus, the effects of CS treatment persisted longer after discontinuation than those of traditional therapy.

A computerized technique was used to measure medial tibiofemoral joint space in patients treated with 800 mg CS

per day or with placebo. After 1 year, tibiofemoral joint space width had decreased significantly in placebo-treated patients but had not changed from the baseline value in the CS treatment group [38]. The aim of this study was to assess the clinical, radiological and biological efficacy and tolerability of the SYSADOA, chondroitin 4- and 6-sulfate (Condrosulf, IBSA, Lugano, Switzerland), in patients suffering from knee osteoarthritis. This was a 1-year, randomized, double-blind, controlled pilot study, which included 42 patients of both sexes, aged 35-78 years with symptomatic knee OA. Patients were treated orally with 800 mg CS per day or with a placebo (PBO) administered in identical sachets. The main outcome criteria were the degree of spontaneous joint pain and the overall mobility capacity. Secondary outcome criteria included the actual joint space measurement and the levels of biochemical markers of bone and joint metabolism. This limited study confirmed that CS was well-tolerated and both significantly reduced pain and increased overall mobility capacity. Treatment with CS was also associated in a limited group of patients with a stabilization of the medial femoro-tibial joint width, measured with a digitized automatic image analyzer, whereas joint space narrowing did occur in placebo-treated patients. In addition, the metabolism of bone and joint assessed by various biochemical markers also stabilized in the CS patients whereas it was still abnormal in the PBO patients. These results confirm that oral chondroitin 4- and 6-sulfate is an effective and safe symptomatic slow-acting drug for the treatment of knee OA. In addition, CS might be able to stabilize the joint space width and to modulate bone and joint metabolism. This is the first preliminary demonstration that a SYSADOA might influence the natural course of OA in humans.

Verbruggen and colleagues [39] evaluated hand radiographs of 119 patients with OA. Thirtyfour patients who received CS 400 mg three times a day were compared with the results of 85 patients who received placebo. Radiographs were performed annually over a 3-year period. In the CS-treatment group, a significant decrease in the number of patients with new erosive OA was seen. A total of 119 patients were included in a randomized, double-blind, placebo-controlled trial in order to assess the S/DMOAD properties in OA of CS (3 x 400 mg/day, Condrosulf IBSA, Lugano, CH). Posteranterior roentgenographies of the interphalangeal (IP) joints were carried out at the start of the study and at yearly intervals. This enabled the investigators to document the radiological progression of the anatomical lesions in the pathological finger joints over a 3-year period. It was shown that the progression of OA in the IP finger joints in an individual can be determined by the evolution of his finger joints through previously described anatomical phases: N (not affected), S (classical OA), J (loss of joint space), E (erosive OA) and R (remodeled joint). Structure/disease-modifying anti-OA drug (S/DMOAD) properties were searched for by assaying the number of patients developing OA in previously normal IP joints (N > S), or progressing through the described anatomical phases of the disease (S > J, S > E, J > E, S > R, J > R, E > R). In the CS group we observed a significant decrease in the number of patients with new erosive OA finger joints. This result is particularly important since OA of the finger joints becomes a clinical problem (pain, functional loss) when S

joints progress to J and especially E phases. During and after these E phases, joints will remodel and show the nodular deformities characteristic of Heberden's and Bouchard's nodes. Treated patients were protected against erosive evolution. The implication of this last study is that CS may have chondroprotective properties in patients with OA.

## ABBREVIATIONS

OA	=	Osteoarthritis
CS	=	Chondroitin sulfate,
PG	=	Proteoglycan
HA	=	Hyaluronan,
NSAID	=	Nonsteroidal antiinflammatory drug
COX	=	Cyclooxygenase

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