THE EVOLUTION OF
CHONDROITIN SULFATE

Volume editor
NICOLA VOLPI

NON-ANIMAL
CHONDROITIN SULFATE
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With a special thanks to Ms Lorena Carboni
THE EVOLUTION OF CHONDROITIN SULFATE
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GLOSSARY
According to EULAR (the European League Against Rheumatism)\cite{1,2}, OARSI (Osteoarthritis Research Society International)\cite{3} and World Health Organization (WHO)\cite{4}, the musculoskeletal or rheumatic diseases are the major cause of morbidity throughout the world, having a substantial influence on health and quality of life, also causing an enormous burden of cost on health systems. Osteoarthritis (OA) is an important example of the more than 150 conditions and syndromes with the common denominators of pain and inflammation. 40% of people over the age of 70 years suffer from OA of the knee, 80% of patients with OA have some degrees of limitation of movement, and 25% cannot perform their major activities of daily living\cite{4}.

Most people do not develop symptoms of OA until significant joint damage has occurred, commonly after age 50-60 years, but there is radiographic evidence for OA in a significant percent of women beginning in the early 40’s\cite{5}. However, this stage already represents a situation where cartilage is heavily damaged leading to a narrowing of the joint space.

Over the last years, there has been an increasing interest on individuals with ageing and their quality of life along with increased healthcare costs for the demographic shift towards higher fractions of elderly people\cite{6}. Today, along with traditional use of drugs to treat the pain of OA, great results are associated with administering chondroprotective bioactive (macro)molecules in the early stages of OA, in order to delay progress and/or to reduce symptoms.

Chondroitin sulfate (CS), a basic natural component of cartilage and synovial fluid, is one of the most effective Symptomatic Slow-Acting Drugs for Osteoarthritis (SYSADOAs) and it is a S/DMOAD (structure/disease modifying anti-osteoarthritis drug) used to provide relief from the pain and inflammation associated with joint degeneration. Its supplementation helps to regenerate the joint structure, leading to reduced pain and increased mobility of the affected joint\cite{7}. The clinical benefits of CS have been established by a large number of human trials and offers an excellent safety and tolerability profile which allows long term administration for OA and joint health.
However, it should be pointed out that all studies have been carried out on quality-controlled products of CS. The respective results can in no way be translated to the heterogeneity of products available over the counter.

CS supplied to the market is extracted from cartilaginous feedstocks using organic solvents, with purification processes that may cause chemical degradation/desulfation and loss of activity along with an uncontrolled and unreliable supply chain of CS raw materials. This poses serious concerns about the quality and the safety of the ingredient.

As a matter of fact, several published studies have reported poor quality of CS present in nutraceuticals\(^\text{[8,9]}\). Finally, today, a new and pioneering non-animal CS characterized by high purity and obtained through a fermentation-derived manufacturing process is available and promise to resolve the long-standing acknowledged problem of poor quality and potential safety issues of animal-derived CS.

Mythocondro\(^\text{®}\) is a revolutionary ingredient, patented and developed by Gnosis using a pharmaceutical approach, to ensure strict quality standards that promises to change completely the CS industry, providing a reliable and reproducible source of CS which definitely solves concerns related to animal derived CS: clinically tested, well characterized and environmentally friendly, it is the first CS suitable for vegetarians and free from restrictions of use related to religious and supply issues.

This book gives an extensive overview of the aetiopathogenesis of OA, of the current database of CS in the treatment of OA and the relevant description and clinical evidence of this new non-animal, fermentation derived CS with relevant effectiveness in joint disease.
JOINT HEALTH AND CHONDROITIN SULFATE
Understanding of joint dysfunction requires some knowledge of normal joint structure, its physiology and the comparison of the diseased state to the physiological situation.

Joints are responsible for movement and stability of the skeleton and are classified based on structure or function. In particular, synovial joints are the only joints that have a space (a synovial cavity filled with fluid) between the adjoining bones and that make possible movements of the opposed articular surfaces without pain, friction with a correct distribution of load across joint tissues. In a healthy joint, the cartilage within the joint serves as a cushion, permitting the bones to rotate, glide and roll upon each other smoothly and easily during activities like walking\textsuperscript{10-12}.

Articular cartilage tissue varies in thickness, cell density, matrix composition, and mechanical properties within the same joint, among joints and among species\textsuperscript{13}. Nonetheless, it consists of the same components and has the same general structure.

It is composed of a dense extracellular matrix (ECM) with a sparse distribution of highly specialized cells called chondrocytes. The ECM is principally composed of water, collagen and proteoglycans (PGs) with other non-collagenous proteins and glycoproteins present in lesser amounts\textsuperscript{14} (Figure 1).

Figure 1. To form articular cartilage, chondrocytes organize collagen, proteoglycans and other components into a unique, highly ordered structure.
Water contributes up to 80% of the wet weight of articular cartilage and its interaction with the matrix macromolecules significantly influences the mechanical properties of the tissue.

PGs are heavily glycosylated structures. In articular cartilage, they represent the second-largest group of macromolecules in the ECM and account for 10% to 15% of the wet weight. PGs consist of a protein core with one or more linear glycosaminoglycan (GAG) chains (CS, keratan sulfate and others) covalently attached [14] (Figure 2).

Maintenance of the articular surface requires turnover of the matrix macromolecules (continual replacement of degraded matrix components) and probably alteration in the matrix macromolecular framework in response to joint use [14].

Figure 2. Structure of a proteoglycan with its CS and keratan sulfate chains and the formation of a macromolecular complex associated with hyaluronic acid.
During the normal biological process of ageing, multiple factors may impact on joint structure (including genetic, developmental, metabolic and traumatic events \[^{[15]}\]) determining an imbalance between the normal coupling of degradation of joint and the synthesis of articular cartilage, ECM and subchondral bone by chondrocytes (Figure 3). Moreover, a loss of chondrocytes due to an increased susceptibility to cell death appears to be important as well\[^{[16-18]}\].

These changes are directly responsible to the development of a typical age-related disorder, the degenerative joint disease also known as OA.

In OA, the gradual loss of matrix PGs and subsequent loss of mechanical integrity is often a slow process believed to take place over decades.

Human articular cartilage is continuously remodeled as a consequence of anabolic and catabolic processes. The chondrocytes in normal adult cartilage maintain a balance between synthesis and degradation of ECM components. In OA, the metabolic activity of the chondrocytes is shifted towards a state where new

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**Figure 3.** The correct definition of OA is a chronic (long-term) condition “characterized by focal areas of loss of articular cartilage within the synovial joints, associated with hypertrophy of the bone (osteophytes and subchondral bone sclerosis) and thickening of the capsule. In this sense it is a reaction of the synovial joints to injury”\[^{[4]}\].
matrix synthesis is outweighed by breakdown of matrix constituents. The result is degeneration and gradual loss of articular cartilage\cite{10}.

The avascular nature of cartilage leaves the chondrocyte as the only mechanism of matrix repair and the isolated nature of the cell severely limits its repair capabilities. The breakdown of the tightly regulated structure of the ECM leads to a weakening of the mechanical properties of the matrix and can progress to the endpoint of total failure.

The pathology of OA involves the whole joint in a disease process that includes focal and progressive hyaline articular cartilage loss with concomitant changes in the bone underneath the cartilage, including development of marginal outgrowths, osteophytes and increased thickness of subchondral bone.

OA can affect all joints of the body but not all localizations have the same consequences for the individual. Some joints are more prone to be affected by OA than others (Figure 4). Exact conditions for the individual joints are sparse, mostly

\textit{Common Joints affected by osteoarthritis}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{common-joints.png}
\caption{Common joints affected by OA.}
\end{figure}
non-representative and differ considerably depending on definition (radiographic vs. symptomatic), age group, sample size, ethnicity and region.

Severe OA of the large joints like the hip or the knee will lead to more dramatic effects such as immobility of the individual and loss of working resources to society\cite{18}.

1.2.1 Etiology of osteoarthritis

OA is believed to be caused by a combination of factors. Due to the long period of painless progression, the disease is rarely diagnosed at an early time point. This makes diagnosing the exact cause in specific patients extremely difficult. Injury due to mechanical stress, although poorly understood, is considered to play a predominant role during initial stages of the disease\cite{19-25}. Biochemical and genetic factors are likely to contribute to the further progression.

The role of genetics in the onset of OA is becoming increasingly playing a larger role than first thought. These studies indicate that some forms of OA may have a genetic component of up to 65\%\cite{26,27}. An up-regulation of proteinase activities, particularly those of metalloproteinases (MMPs), has been implicated in the disease as the major cause of increased matrix catabolism\cite{28-32}. The degradation of structural macromolecules like PGs and collagens leads to depletion of the most important building blocks of the ECM. Under these circumstances, the absence of adequate replenishment becomes a main issue.

The onset of the disease is believed to be a loss of GAGs from the upper layers of the cartilage. This is then followed by a gradual breakdown of collagen fibers with subsequent fibrillation of the cartilage surface. Once this point has been reached the progression of the disease becomes more rapid and irreversible. The cartilage matrix is continually degraded, deep fissures are generated and finally the matrix is removed completely exposing the underlying bone. It has also been demonstrated that there is an increased rate of apoptosis within OA cartilage. As the chondrocyte is an isolated cell, the death of a cell leaves a zone of cartilage which has no means of maintenance.
1.2.2 Role of the chondrocyte in cartilage destruction

Chondrocytes represent the only cell type in hyaline cartilage. These cells are responsible not only for the generation of ECM during growth and development, but also for the maintenance of tissue homeostasis during adult life. The chondrocyte in mature articular cartilage exhibits virtually no mitotic activity and a very low rate of matrix synthesis and degradation. However, chondrocytes even from old individuals are able to respond to given stimuli by showing increased activity.

In early OA, structural changes in the ECM induce chondrocyte proliferation (clonal growth), a stimulated collagen and PG biosynthesis corresponding to a repair attempt, and an increased production of catabolic cytokines and matrix-degrading proteinases[28-33].

The capacity of chondrocytes to degrade constituents of the ECM depends on their ability to synthesize and secrete proteinases (Table 1).

There are more than 20 known MMPs divided into four categories: collagenases, stromelysins, gelatinases and membrane bound (Table 1).

<table>
<thead>
<tr>
<th>MMPs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>MMP-1, MMP-8, MMP-13</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>MMP-2, MMP-9</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>MMP-3, MMP-7, MMP-10, MMP-11</td>
</tr>
<tr>
<td>Membrane type</td>
<td>MMP-14, MMP15, MMP16, MMP17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aggreccanases</th>
<th>ADAM-TS4, ADAM-TS5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other proteinases</td>
<td></td>
</tr>
<tr>
<td>Elastase</td>
<td></td>
</tr>
<tr>
<td>Cathepsins</td>
<td>Cathepsin B, D, G, L</td>
</tr>
<tr>
<td>Proteinases of the coagulation system</td>
<td>tPA, uPA, plasmin</td>
</tr>
</tbody>
</table>

Table 1. Proteinases involved in the degradation of cartilage matrix.
This list is representative, not comprehensive.

Under normal conditions, the controlled activity of these enzymes are necessary for appropriate morphogenesis and tissue remodeling (Figure 5). Regulation of proteinase activity occurs at three different levels: synthesis and secretion, activation of latent enzyme and inactivation by proteinase inhibitors. During degenerative joint diseases, such as OA, the expression and production of proteinases is increased. MMPs are prominent and appear to play several important roles in the pathological destruction of cartilage[28-32,34-37].

![CARTILAGE IN NORMAL CONDITIONS: BALANCE BETWEEN TROPHIC AND LITHIC PROCESSES](image)

**Figure 5.** Normal cartilage: balance between biosynthetic and catabolic processes.

### 1.2.3 Role of biomechanics and load

While the role of mechanical forces on OA has long been speculated, it is only over the last few years that data has appeared supporting this hypothesis. The increased incidence of OA in overweight patients may be due to the increased mechanical forces being applied. Body mass index (BMI) and obesity have been shown to be linked to the incidence of OA[22-23].
In fact, it has been shown that the BMI at 20-29 years was the most predictive of future OA incidence\[^{25}\] , indication that OA progresses over a number of decades.

Joint injury has been shown to be a risk factor for knee OA\[^{22,23}\] and a number of studies have demonstrated a link between occupations involving heavy lifting or repetitive movements on the incidence of OA\[^{38-42}\].

*In vitro* studies have been used to elucidate the early events after mechanical damage\[^{43-46}\]. It has been shown in cartilage explants that within hours of an excessive mechanical insult there is a large, enzyme independent, release of GAGs into the surrounding medium\[^{46}\] and excessive load increases activation and expression of MMPs in explant cultures\[^{47-51}\]. This would suggest that injury modulates the mechanism of GAG turnover\[^{46}\]. Short term responses to mechanical load and injury include cell death by both necrosis and more commonly apoptosis\[^{44,45}\]. This loss of cells may have long term effects in the maintenance of the surrounding matrix. Once the disease has progressed OA, chondrocytes have a differential response to mechanical load than normal chondrocytes\[^{52,53}\]. Whether this altered response is a cause of the disease or a later consequence is still unknown.

The risk factors for OA can be separated in three groups\[^{54}\]: systemic, intrinsic and extrinsic factors (Table 2).

<table>
<thead>
<tr>
<th>Systemic Factors</th>
<th>Intrinsic factors (anatomically or physiologically pertain to a joint)</th>
<th>Extrinsic factors (somehow acting on a joint)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Higher age</td>
<td>• Deformities</td>
<td>• Obesity</td>
</tr>
<tr>
<td>• Female gender</td>
<td>• Injuries/damages</td>
<td>• Muscle weakness</td>
</tr>
<tr>
<td>• Race</td>
<td>• Malalignment</td>
<td>• Repetitive overuse (occupational or sports)</td>
</tr>
<tr>
<td>• Inheritance</td>
<td>• Laxity</td>
<td></td>
</tr>
<tr>
<td>• Possibly nutritional factors</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 2. Risk factors for the development of OA.*
All these factors may differ in extent from one joint to another. Some, like inheritance, may only multiply a selection of those among in- and extrinsic factors. For women, weight accounts for more OA than any other known factor, for men’s knee OA overweight ranks second after injury as predisposing factor\cite{55-57}.

1.2.4 Mediators of inflammation in osteoarthritis

It is generally accepted that inflammatory mediators including cytokines, prostanoids and nitric oxide (NO) induce cartilage degradation in disorders such as rheumatoid arthritis (RA) \cite{58 and 59}. In that case, the inflammation primarily takes place in the synovial membrane and the destruction of cartilage is a secondary event. In synovial cells, reactions towards components released from cartilage into the synovial fluid may contribute to the disease progression\cite{60,61} but the major pathogenic processes are localized within the cartilage itself\cite{62,63}. Chondrocytes in OA affected cartilage display enhanced and coordinated expression of proinflammatory cytokines and the enzyme responsible for NO production\cite{64}. Collectively, evidence is accumulating that mediators of inflammation acting in an autocrine/paracrine fashion within the cartilage play a primary role in the pathogenesis of OA\cite{60-65}.

Proinflammatory cytokines include interleukins (IL), tumor necrosis factors, interferons and colony-stimulating factors. Although these factors were identified originally as secreted products of immune cells that modulate the function of other cells of the immune system, many of them have effects on nonimmune cells like fibroblasts and chondrocytes. Among the proinflammatory cytokines, IL-1β and TNF-α appear most directly involved in the pathological processes of OA\cite{66}.

Anyway, it appears that cytokine networks with a considerable degree of complexity influence the metabolic state of chondrocytes. Evidences have accumulated that proinflammatory cytokines are important in the pathogenesis of OA and a large number of \textit{in vitro} studies have been carried out in
order to examine in detail the influence of these factors on chondrocytes. IL-1 has been found to induce catabolic responses in many different ways. Human articular chondrocytes, when stimulated by IL-1, dramatically increase the expression of matrix-degrading proteinases like MMP-1, -2, -3, -7, -8, -9, -13\[37,67-70\]. Moreover, IL-1 strongly inhibits biosynthesis of cartilage PG and collagens\[71,72\].

More recently, it has also been discovered that IL-1 may play a role in anabolic responses in human knee cartilage from both normal and OA patients\[73\]. This study showed that IL-1 and TNF-\(\alpha\), both normally associated with inflammatory responses, are able to upregulate the bone morphogenetic protein-2 in a dose dependent fashion and IL-1 is also associated with a reduction in GAG synthesis (Figure 6).
OA is a global issue affecting all known cultures and ethnicities. Detailed information for incidence and prevalence are sparse, not only because of limitations of available research resources, but also related to methodological and diagnostic difficulties. Not all races are affected equally when differentiating by anatomic criteria. Anyway, OA has a huge incidence and economic impact (Table 3).

<table>
<thead>
<tr>
<th>Country</th>
<th>Cost estimate</th>
<th>% of gross domestic product</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>US$ 69.8-84.4 billions</td>
<td>0.8-1.0</td>
<td>1997</td>
</tr>
<tr>
<td>Australia</td>
<td>AUS$ 0.94 billions</td>
<td>0.17</td>
<td>1993-94</td>
</tr>
<tr>
<td>Great Britain</td>
<td>GBE 0.22 billions</td>
<td>0.35</td>
<td>1998-99</td>
</tr>
<tr>
<td>France</td>
<td>EUR 1.5 billions</td>
<td>0.18</td>
<td>1998</td>
</tr>
<tr>
<td>Germany</td>
<td>US$ 6.7 billions</td>
<td>0.32</td>
<td>1994</td>
</tr>
</tbody>
</table>

Table 3. Perspective of country-specific estimates of OA costs.

African-American women seem to be more prone to OA of the knee than Caucasian women\(^{[74]}\). Paleopathological studies suggest that OA has been with mankind from the beginnings as traces of OA are revealed by many skeletons since Paleolithic times\(^{[75-77]}\).

OA is particularly prevalent among older people, the number of whom is predicted to increase in all countries. Worldwide estimates suggest that 9.6% of men and 18.0% of women from age 60 have symptomatic OA\(^{[78]}\). 40% of people over the age of 70 years suffer from OA of the knee, 80% of patients with OA have some degrees of limitation of movement and 25% cannot perform their major activities of daily living.

Since OA is a disease whose prevalence increases with age, it will become even more frequent in the future as the proportion of the population above 70 years of age is expected to increase dramatically\(^{[79,80]}\). Moreover, with the observed increase of obesity in western populations an over-proportionate rise in the prevalence of knee, hip and hand OA can be expected\(^{[81]}\).

OA does not per se reduce life expectancy. But comorbidities, predisposing fac-
tors (obesity) and side effects of medications (in particular non-steroidal anti-inflammatory drugs) themselves have an elevated risk of death. In 2000, 13,000 years of life were lost due to premature death because of OA worldwide.

Finding cost effective treatment modalities are among the most important challenges for science and politics in the near future. It is currently uncertain whether measures exist to effectively prevent OA. Great hopes are associated with administering bioactive (macro)molecules in the early stages of OA in order to delay progress or to reduce symptoms. It is not clear yet what economic impact such medication of typically long duration will have. The core question is whether its direct costs will be outweighed by savings on surgery, analgesics and anti-inflammatory medications.

Current treatments focus mainly on pain relief, although a growing number of experimental treatments are under investigation with the aim of slowing or reversing the progression of the disease (with S/DMOAD). The only reproducibly effective treatment is total replacement of late stage OA joints with prosthetic joints.

More and more data have been cumulated indicating treatment potentials for pain relieve, functional improvement and slowing of destructive processes, most often based on studies applying natural constituents of cartilage to animals and humans.

Among others, the treatment concept using CS in OA appears to be one of the most beneficial strategies both with regard to improvement of symptoms and disease modification. In several published studies, CS has in fact demonstrated to have properties of a so called symptomatic slow acting drug for the treatment of OA (SYSADOA) and to own disease modifying properties in the long-term treatment of OA (so called DMOAD).

Along with these epidemiological and clinical findings research regarding the design of clinical trials has been strengthened. CS in particular was studied extensively with regard to pharmacokinetics and mode of action. Several lines of research strongly point to different, perhaps complementary ways how CS appears to re-establish the disturbed metabolism of cartilage in OA.
1.4 CHONDROITIN SULFATE AND ITS ROLE IN MANAGEMENT OF OSTEOARTHRITIS

CS is a natural sulfated GAG which play a significant role in biological processes as it is abundantly distributed in humans, other mammals and invertebrates. Based on its structural diversity in chain length and sulfation patterns, CS provides specific biological functions in cell adhesion, morphogenesis, neural network formation and cell division.

GAGs are a family of linear, complex, polydisperse natural polysaccharides represented also by hyaluronic acid, keratan sulfate, dermatan sulfate (DS), heparan sulfate/heparin. With the exception of keratan sulfate, they are composed of alternating copolymers of uronic acids and amino sugars, and their structures are commonly represented by typical disaccharide sequences. GAGs are usually found covalently linked to protein in the form of PGs and fibrous proteins, including collagen, elastin, fibronectin and laminin having both structural and adhesive functions.

CS, in particular, is composed of alternate sequences of D-glucuronic acid and differently sulfated residues of N-acetyl-D-galactosamine linked by $\beta^1,3$ bonds. Depending on the disaccharide nature, CS with different carbohydrate backbones are known (Figure 7).

\[
\begin{align*}
R_1 &= R_2 = R_3 = H: \text{nonsulfated chondroitin} \\
R_1 &= SO_3^- \text{ and } R_2 = R_3 = H: \text{chondroitin-4-sulfate, CSA} \\
R_2 &= SO_3^- \text{ and } R_1 = R_3 = H: \text{chondroitin-6-sulfate, CSC} \\
R_2 &= R_3 = SO_3^- \text{ and } R_1 = H: \text{chondroitin-2,6-disulfate, CSD} \\
R_1 &= R_2 = SO_3^- \text{ and } R_3 = H: \text{chondroitin-4,6-disulfate, CSE} \\
R_1 &= R_3 = SO_3^- \text{ and } R_2 = H: \text{chondroitin-2,4-disulfate, CSB} \\
R_1 &= R_2 = R_3 \text{ and } SO_3^-: \text{trisulfated chondroitin}
\end{align*}
\]

Figure 7. Structures of the disaccharides forming chondroitin sulfate. Minor percentages of very rare and peculiar disaccharides may also have a sulfate group in position C3 of the glucuronic acid.
However, even if the known CS samples are mainly composed of various percentages of chondroitin-4-sulfate (CSA) and chondroitin-6-sulfate (CSC), disaccharide units (monosulfated in position 4 and monosulfated in position 6 of the N-acetyl-D-galactosamine) and disaccharides with a different number and position of sulfate groups can be located, in various percentages, within the polysaccharide chains (Figure 7). In fact, different kinds of

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CS Bovine</th>
<th>CS Porcine</th>
<th>CS Chicken</th>
<th>CS Shark</th>
<th>CS Raja</th>
<th>CS Squid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular mass</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MWh (kDa)</td>
<td>12±17</td>
<td>9±14</td>
<td>8±13</td>
<td>25±40</td>
<td>27±34</td>
<td>60±80</td>
</tr>
<tr>
<td>MWw (kDa)</td>
<td>20±26</td>
<td>14±20</td>
<td>16±21</td>
<td>50±70</td>
<td>50±70</td>
<td>80±120</td>
</tr>
<tr>
<td>Dispersity</td>
<td>1.8±2.2</td>
<td>1.4±1.8</td>
<td>1.6±2.0</td>
<td>1.0±2.0</td>
<td>1.2±2.5</td>
<td>0.8±1.3</td>
</tr>
<tr>
<td><strong>Disaccharides</strong></td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>∆Di-0s</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>∆Di-6s</td>
<td>33</td>
<td>14</td>
<td>20</td>
<td>44</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td>∆Di-4s</td>
<td>61</td>
<td>80</td>
<td>72</td>
<td>32</td>
<td>43</td>
<td>50</td>
</tr>
<tr>
<td>∆Di-2,6dis</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>18</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>∆Di-4,6dis</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>2</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>∆Di-2,4dis</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Charge Density</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.90±0.96</td>
<td>0.92±0.96</td>
<td>0.90±0.94</td>
<td>1.15±1.25</td>
<td>1.08±1.20</td>
<td>1.00±1.20</td>
<td></td>
</tr>
<tr>
<td>4s/6s ratio</td>
<td>1.50±2.00</td>
<td>4.50±7.00</td>
<td>3.00±4.00</td>
<td>0.45±0.90</td>
<td>1.00±1.40</td>
<td>2.50±4.00</td>
</tr>
<tr>
<td>Reference(s)</td>
<td>Volpi N.</td>
<td>Volpi N.</td>
<td>Volpi N.</td>
<td>Volpi N.</td>
<td>Volpi N.</td>
<td>Volpi N.</td>
</tr>
<tr>
<td></td>
<td>J Pharm</td>
<td>J Pharm</td>
<td>J Pharm</td>
<td>J Pharm</td>
<td>J Pharm</td>
<td>J Pharm</td>
</tr>
</tbody>
</table>

Table 4. Main CS disaccharides from various species, organ and tissues.
CS sulfation may be present in the CS backbone in various percentages in relation to specific animal sources (Table 4)\textsuperscript{[86]}.

CS is generally found in all mammalian connective tissues, especially in the cartilage, skin, blood vessels, ligaments, tendons with different kind of sulfation\textsuperscript{[87]}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Sturgeon bone} & \textbf{Aorta PGs} & \textbf{Fetal bovine muscle, ileum, kidney, lung Rabbit bone marrow} & \textbf{Platelets} & \textbf{Human plasma} \\
\hline
25±30 & nd & nd & nd & nd \\
35±40 & nd & nd & nd & ~15 \\
1.0+1.5 & nd & nd & nd & nd \\
\hline
7 & 0 & nd & 0 & 40±60 \\
55 & 95±100 & 100 & traces & 1±5 \\
38 & 0±5 & traces & >98 & 60±40 \\
0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 \\
0 & 0.90±0.95 & 0.98±1.02 & 0.98±1.02 & 0.98±0.60 \\
0 & 0.40±0.90 & <0.1 & <0.1 & >45 \\
\hline
\end{tabular}
\caption{Main CS disaccharides from various species, organ and tissues.}
\end{table}
1.4.1 Biological functions of chondroitin sulfate

The effect of CS in patients with OA is possibly the result of the stimulation of the synthesis of PGs and the decrease in catabolic activity of chondrocytes by inhibiting the synthesis of proteolytic enzymes and other factors that contribute to cartilage matrix damage and cause the death of these cells (Figure 8).

In addition to its conventional structural roles in the composition of ECM and formation of organs and tissues, such as cartilages and bones, accumulated evidence implies that CS chains fulfil important biological functions in inflammation, cell proliferation, differentiation, migration, tissue morphogenesis, organogenesis, infection and wound repair\(^{88-91}\).

These effects are related to the capacity of CS (and CS-PGs) to interact with a wide variety of molecules including (but not limited to) matrix molecules, growth factors, protease inhibitors, cytokines, chemokines, adhesion molecules and pathogen virulence factors via aspecific/specific saccharide domains within the
chains. Along with aspecific interactions based on both numerous negative charges related to sulfate and carboxyl groups and to its long chains and on particular functional domain structures which are formed by combinations of the various disaccharide units (see Figure 7), CS may participate in specific binding to bioactive molecules (Table 5).

<table>
<thead>
<tr>
<th>CS-Binding proteins</th>
<th>CS bound</th>
<th>Biological effects related to the binding protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ECM Components</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II collagen</td>
<td>CS</td>
<td>Primary OA</td>
</tr>
<tr>
<td>Type V collagen</td>
<td>CS</td>
<td>Tuberous sclerosis</td>
</tr>
<tr>
<td><strong>Growth factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-2, -10, -16, -18</td>
<td>CS type E</td>
<td>Wound healing, angiogenesis-related diseases</td>
</tr>
<tr>
<td>KGF/FGF-7</td>
<td>CS</td>
<td>Tissue repair, cancer</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>CS</td>
<td>Arteriosclerosis, liver cancer, wound healing</td>
</tr>
<tr>
<td>MK</td>
<td>CS type E</td>
<td>Cancer</td>
</tr>
<tr>
<td>PTN/HB-GAM</td>
<td>CS type E</td>
<td>Cancer</td>
</tr>
<tr>
<td>PDGF</td>
<td>CS</td>
<td>Arteriosclerosis, malignant tumor</td>
</tr>
<tr>
<td>VEGF</td>
<td>oversulfated CS</td>
<td>Diabetic retinopathy, solid tumor, RA</td>
</tr>
<tr>
<td>GDNF</td>
<td>CS type E</td>
<td>Extensive aganglionosis</td>
</tr>
<tr>
<td>BDNF</td>
<td>CS type E</td>
<td>Autism</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>CS</td>
<td>Antiviral and antitumor actions, receptor for INF-γ</td>
</tr>
<tr>
<td>IL-8</td>
<td>CS</td>
<td>Arthritis, sepsis, acute, nephritis, etc.</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CS</td>
<td>Myeloma bone disease</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CS</td>
<td>Atherosclerosis, multiple sclerosis</td>
</tr>
<tr>
<td>SLC</td>
<td>oversulfated CS</td>
<td>Inflammation</td>
</tr>
<tr>
<td>IP-10</td>
<td>oversulfated CS</td>
<td>Inflammation, cancer</td>
</tr>
<tr>
<td>SDF-1β</td>
<td>oversulfated CS</td>
<td>Hematopoiesis</td>
</tr>
<tr>
<td>PF4</td>
<td>oversulfated CS</td>
<td>Hematopoiesis, angiogenesis</td>
</tr>
<tr>
<td><strong>Cell adhesion molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-44</td>
<td>oversulfated CS</td>
<td>Inflammation, malignant tumor</td>
</tr>
<tr>
<td>L-selectin</td>
<td>oversulfated CS</td>
<td>Leukocyte adhesion deficiency</td>
</tr>
<tr>
<td>P-selectin</td>
<td>oversulfated CS</td>
<td>Inflammation, thrombosis</td>
</tr>
<tr>
<td><strong>Proteases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocyte elastase</td>
<td>CS</td>
<td>Inflammation, lung emphysema, clotting disorders</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>CS type A</td>
<td>Collagenolytic activity</td>
</tr>
<tr>
<td>Matrix metalloproteinase-2</td>
<td>CS</td>
<td>Tumor cell invasion and metastasis</td>
</tr>
<tr>
<td><strong>Virus protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycoprotein C</td>
<td>CS type E</td>
<td>Herpes simplex virus infection</td>
</tr>
</tbody>
</table>

Table 5. Binding of CS to various biomolecules.
1.4.2. Preclinical studies of chondroitin sulfate

The results of the several pharmacokinetic studies in rats, dogs and humans present clear evidence that exogenous CS is absorbed following oral administration as a high molecular mass polysaccharide together with derivatives resulting from a partial depolymerization and/or desulfation of the parent compound.

As a consequence, CS has a systemic bioavailability and can exhibit pharmacological actions in the human target tissues like joint cartilage and synovial fluid.

1.4.2.1 Intestinal absorption of chondroitin sulfate

The absorption of sulfated GAGs orally administered remains a controversial issue since longer time, due to the difficulty in accepting that molecules with high molecular mass and charge density can pass the gastric and intestinal mucosa. In contrast to glucosamine sulfate, for example, that is absorbed easily, CS is a much larger molecule which was long discussed not to be absorbed intact through the intestinal wall and into blood and joints. If the molecule is too large to pass intact through the intestinal wall, bioavailability would be restricted to the intestinal wall cells with which it is in direct contact.

As a consequence, the systemic availability would be low in terms of penetrating as such into the blood and joints and possibly limiting for the clinical efficacy of CS.

Therefore, several studies have dealt with the absorption and metabolic fate of CS, following oral administration in animals and humans using different test methods (Table 6).

These results confirm that CS with high molecular mass can be absorbed orally. The differences in the absorption and bioavailability of the various CS formulations are strongly influenced by the structure and characteristics, such as molecular mass, charge density and clusters of disulfated disaccharides, of the parental molecules.
<table>
<thead>
<tr>
<th>Model</th>
<th>Drug</th>
<th>Methodology</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Partially depolymerized</td>
<td>Radioactivity</td>
<td>A tropism of the radioactivity towards GAGs-rich tissues is observed. The presence CS Bovine of CS greater than 4000 Da in plasma, synovia and cartilage after oral and intramuscular administrations may explain its chondroprotective effect</td>
<td>[92]</td>
</tr>
<tr>
<td>Human, Rat and Dog</td>
<td>CS Bovine</td>
<td>Uronic acid determination</td>
<td>The absolute bioavailability following oral administration is 13.2%. A peak of oligo- and polysaccharides with a molecular weight lower than 5000 Da derived from partial digestion of exogenous CS is also present in plasma</td>
<td>[93]</td>
</tr>
<tr>
<td>Human</td>
<td>CS Bovine</td>
<td>Radioactivity</td>
<td>After 24 h, radioactivity was higher in the rat intestine, liver, kidneys, synovial fluid and cartilage than in other tissues. Oral CS administration determined a significant increase of plasma concentration as compared with predose value over a full 24 h period</td>
<td>[94]</td>
</tr>
<tr>
<td>Human</td>
<td>CS Bovine</td>
<td>CS disaccharides quantification</td>
<td>Orally administered CS is absorbed as a high molecular mass polysaccharide together with derivatives resulting from a partial depolymerization and/or desulfation</td>
<td>[95]</td>
</tr>
<tr>
<td>Human</td>
<td>CS from shark</td>
<td>CS disaccharides quantification</td>
<td>CS structure and characteristics, such as molecular mass, charge density and cluster of disulfated disaccharides, strongly influence oral absorption and bioavailability</td>
<td>[96]</td>
</tr>
<tr>
<td>Horse</td>
<td>Low molecular weight CS</td>
<td>CS disaccharides quantification</td>
<td>Low molecular weight CS is orally absorbed</td>
<td>[97]</td>
</tr>
<tr>
<td>Different parts of the gastro-intestinal (GI) tract</td>
<td>CS Bovine</td>
<td>Radioactivity</td>
<td>A small amount of CS may cross the upper intestine intact but in the distal GI tract the molecule is effectively degraded, presumably by the enzymes in the intestinal flora, and adsorbed as monosaccharides, disaccharides and oligosaccharides</td>
<td>[98]</td>
</tr>
</tbody>
</table>

Table 6. Oral absorption of chondroitin sulfate.
1.4.2.2 Bioavailability, distribution and target tissue orientation of chondroitin sulfate (animal and human studies)

A suitable systemic bioavailability and even more a preferred distribution to the target site/tissues are very important factors for a sufficient clinical efficacy of CS. Several studies have demonstrated that the substance reaches the joint tissues in appropriate time and concentrations\(^{[94]}\) (Figure 9).

![Graph showing distribution of radioactivity in rat tissues after oral administration of 3H-CS](image)

**Figure 9. Distribution of radioactivity in rat tissues after oral administration of 3H-CS\(^{[94]}\).**

1.4.3 Mode of action of chondroitin sulfate in *in vitro* and *in vivo* models

1.4.3.1 Anti-inflammatory effect of chondroitin sulfate

Some pharmacological activities of CS in rats compared with the anti-inflammatory properties of ibuprofen and indomethacin have been reported by Ronca et al.\(^{[99]}\). The results present evidence that CS administered per os has an anti-inflammatory activity comparable to that of ibuprofen and indomethacin.
CS was found to decrease significantly the granuloma formation and the cell migration and lysosomal enzyme release in the carrageenan pleurisy. Compared with nonsteroidal anti-inflammatory drugs (indomethacin, ibuprofen), CS appears to be more effective on cellular mechanisms of inflammation than on edema formation itself. These results confirmed earlier data reported by Conte et al.\textsuperscript{[100]}.

To get more information on the mechanisms of the anti-inflammatory effect of CS, the effects of partially depolymerised (3 to 15 kD) and desulfated fragments of the drug on human leukocytes were also investigated by Ronca et al.\textsuperscript{[99]}. CS and its fractions, which inhibit 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.

As previously reported, articular damage and synovitis are secondary to local increase of pro-inflammatory cytokines, enzymes with proteolytic activity and enzymes with pro-inflammatory activity (Figure 10).

\textbf{Figure 10.} \textit{CS decreases the synthesis of proteolytic and proinflammatory enzymes and of proinflammatory cytokines.}
Enhanced expression of these proteins in chondrocytes and in synovial membrane appears associated to the activation and nuclear translocation of nuclear factor-kB (NF-kB)\(^{101}\). CS reduces NF-kB nuclear translocation inducing an anti-inflammatory effect at the chondral and synovial levels.

These mechanisms are important for severity and time course of inflammatory reactions giving an insight into the pharmacodynamic mechanisms of the anti-inflammatory and chondroprotective actions of CS, which are the basis for the clinical efficacy of this drug demonstrated in a number of clinical trials in patients with OA (see below).

Finally, it is noteworthy that CS produces no adverse effects on the stomach, platelets and kidneys which is advantageous compared to the usual non-steroidal anti-inflammatory drugs.

1.4.3.2 Other effects of chondroitin sulfate

CS is an inhibitor of extracellular proteases involved in the metabolism of connective tissues and, in addition to its anti-inflammatory effect, CS stimulates \textit{in vitro} PG production by chondrocytes, inhibits cartilage cytokine production and induces apoptosis of articular chondrocytes. CS also increases the intrinsic viscosity of the synovial liquid and, in bones, it accelerates the mineralization process and repair (Figure 11)\(^{102}\). All these data suggest that CS play a role in articular and bone metabolism by controlling cartilaginous matrix integrity and bone mineralization.

Moreover, due to its capacities, there is preliminary evidence showing that in human beings, CS may be of benefit in other diseases where inflammation is an essential marker, such as psoriasis and atherosclerosis. Furthermore, the review of the literature suggests that CS might also be of interest for the treatment of other diseases with an inflammatory and/or autoimmune character, such as inflammatory bowel disease, degenerative diseases of the central nervous system and stroke, multiple sclerosis and other autoimmune diseases (Figure 12)\(^{101}\).
THE EVOLUTION OF CHONDROITIN SULFATE

Inhibitory effects on extracellular proteases
Anti-inflammatory effects
Decrease in clinical functional symptoms in experimental arthritis

Pharmacological properties of chondroitin

CHONDROCYTES
Stimulation of the production of PGs
Reduction of apoptosis
Blockade of the TNF-α receptor

BONES
Increase in the calcium pool
Promotion of in vitro mineralization
Increase in the rate of bone repair

Figure 11. Effects of CS on the osteoarticular system.

Chondroitin sulfate

↓ NF-κB
↓ IL-1β
↓ COX-2
↓ PLA2

↓ TNF-α

Alzheimer, disease
Amyotrophic lateral sclerosis
Atherosclerosis
Crohn’s disease
Diabetes type I
Multiple sclerosis
Parkinson’s disease
Psoriasis
Rheumatoid arthritis
Systemic lupus erythematosus
Ulcerative colitis

Figure 12. CS may be of benefit for multiple autoimmune diseases.
1.4.4 Efficacy of chondroitin sulfate

Pre-clinical and clinical studies have been performed to establish CS dose regimen, safety and efficacy.

Animal models of OA and RA are useful tools for studying joint pathogenic processes. Adjuvant arthritis is one of the most widely used models. Rat adjuvant arthritis is an experimental model of polyarthritis that has been widely used to test numerous anti-arthritis agents either under preclinical or clinical investigation\(^\text{[103]}\).

Several meta-analyses have also presented a lot of evidence that CS must be considered as a SYSADOA for the treatment of OA. The evaluation of clinical trials which were conducted with a sufficient quality have demonstrated that the efficacy of CS treatment is consistent for pain and functional outcomes and have shown in general moderate to large effects for the therapy of OA symptoms. Results concerning the disease-modifying potential of CS (DMOAD) are available but relatively sparse and need further confirmation. Convincing results were obtained from studies using prescription medicines of appropriate quality.

1.4.4.1 Pre-clinical efficacy in animal models

While no animal models mimic perfectly the condition of human diseases, induced arthritis models are easily reproducible, well defined and have proven to be useful for the development of new therapies for arthritis, as exemplified by cytokine blockade therapies. Animal models have been extensively used for pharmaceutical testing, which means that a large amount of data is available for comparison with humans\(^\text{[103]}\). Because of these factors, the adjuvant arthritis animal model along with collagen induced arthritis have been used in several studies to evaluate CS efficacy (Table 7).
1.4.4.2 Establishment of dose regimen

The dose-dependency of the clinical efficacy of CS has been evaluated by a prospective, randomized, double-blind short-term study\textsuperscript{[110]} with the dosages 200 mg, 800 mg, 1200 mg and placebo over a three-month treatment period in patients with femoro-tibial OA. This study demonstrated the significant superiority of the two higher doses of CS, namely 800 and 1200 mg, as compared to placebo treatment and to the lower dose of CS. This

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Biological effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjuvant arthritis</td>
<td>Inhibition of induced arthritis</td>
<td>[104]</td>
</tr>
<tr>
<td>Chymopapain-induced cartilage degradation</td>
<td>Significantly higher cartilage proteoglycan content in CS treated animals</td>
<td>[105]</td>
</tr>
<tr>
<td></td>
<td>Protective effect on the damaged cartilage</td>
<td></td>
</tr>
<tr>
<td>Type II collagen induced arthritis</td>
<td>Reduction of arthritis index and serum anti-collagen II antibody titer in a dose-dependent manner</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Significant inhibition of hind paw edema, synovitis and destruction of the articular cartilage</td>
<td></td>
</tr>
<tr>
<td>Type II collagen induced arthritis</td>
<td>Significant reduction of hind paw edema, anti-type II collagen antibody and TNF-α</td>
<td>[107]</td>
</tr>
<tr>
<td>Atherosclerosis aggravated by chronic-antigen-induced arthritis</td>
<td>CS administration reduced CRP and IL-6</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>CS administration reduced the nuclear translocation of NF-kB</td>
<td></td>
</tr>
<tr>
<td>Adjuvant arthritis</td>
<td>CS reduced the severity of arthritis and oxidative stress</td>
<td>[109]</td>
</tr>
<tr>
<td></td>
<td>CS improved total antioxidant status and GGT activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CS reduced the production of pro-inflammatory cytokines, CRP, phagocytic activity and the intracellular oxidative burst of neutrophils</td>
<td></td>
</tr>
</tbody>
</table>
difference became evident starting from day 42 and was maintained until the end of the study (day 90). No difference was found between CS 800 mg and CS 1200 mg.

Bourgeois et al.\textsuperscript{[111]} compared the efficacy and tolerability of an oral treatment with CS 1200 mg/day, a treatment with CS capsules at a dose of 3 x 400 mg/day versus placebo in patients with mono or bilateral knee OA. As a result, the single dose 1200 mg/day did not differ from the results of 3 x 400 mg/day for all clinical parameters taken into consideration.

1.4.4.3. Clinical efficacy and safety of chondroitin sulfate in humans

Extensive literature about clinical trials is available on the capacity of CS to act as a SYSADOA and DMOAD in humans affected by OA (Figure 13).

![Figure 13. Human clinical trials available on CS.](image)

All obtained results have been analyzed in ten meta-analysis studies (Table 8)\textsuperscript{[112]}.
<table>
<thead>
<tr>
<th>Year</th>
<th>Study</th>
<th>Agent</th>
<th>Size</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>The efficacy of CS in the treatment of OA was evaluated</td>
<td>CS</td>
<td>7 trials of 372 patients taking CS were enrolled in the meta-analysis</td>
<td>CS may be useful in OA as a SYSADOA. Further investigations in larger cohorts of patients are needed</td>
</tr>
<tr>
<td>2003</td>
<td>To assess the structural and symptomatic efficacy of oral CS/GlcN in knee OA</td>
<td>CS and Glucosamine (GlcN)</td>
<td>An exhaustive systematic research of randomized, placebo-controlled clinical trials published between 1980 and 2002. 1775 patients were analyzed in 15 selected studies</td>
<td>This study demonstrates the indistinguishable symptomatic efficacies for both compounds</td>
</tr>
<tr>
<td>2007</td>
<td>To determine the effects of CS on pain in patients with OA.</td>
<td>CS</td>
<td>20 trials, 3846 patients; contributed to this meta-analysis</td>
<td>Heterogeneity among the trials made initial interpretation of results difficult</td>
</tr>
<tr>
<td>2007</td>
<td>To determine the short-term pain-relieving effects by performing a systematic review of randomized placebo-controlled trials</td>
<td>CS (and other 6 pharmacological agents for OA pain)</td>
<td>In total, 14060 patients in 63 trials were evaluated</td>
<td>CS had maximum mean efficacies at 1-4 weeks</td>
</tr>
<tr>
<td>2008</td>
<td>A meta-analysis to assess the efficacy of CS as an S/DMOAD agent for knee OA</td>
<td>CS</td>
<td>A MEDLINE search was conducted from 1996 through 2007. There was no evidence of heterogeneity across the trials</td>
<td>CS is effective in patients with OA of the knee. CS may have a role as an S/DMOAD agent in the management of patients with knee OA</td>
</tr>
<tr>
<td>2008</td>
<td>A MEDLINE database search was conducted for appropriate meta-analyses published between 1997 and 2007</td>
<td>CS</td>
<td>Five meta-analyses that limited their analysis to randomized controlled trials comparing CS with placebo</td>
<td>CS has a slight to moderate efficacy in the symptomatic treatment of OA, with an excellent safety profile</td>
</tr>
<tr>
<td>2010</td>
<td>The effect of GlcN, CS or their combination on joint pain and on radiological progression of disease in OA of the hip or knee</td>
<td>CS and GlcN</td>
<td>Direct comparisons within trials were combined with indirect evidence from other trials by using a Bayesian model that allowed the synthesis of multiple time points</td>
<td>GlcN, CS and their combination do not reduce joint pain or have an impact on narrowing of joint space</td>
</tr>
</tbody>
</table>

Table 8. *Meta-analysis studies available on CS*[^112^].
The first meta-analysis study was performed in 2000\cite{113}. Several other meta-analysis of clinical trials have been published since that first report (Table 8). CS, alone or in combination with other agents, was found able to reduce joint pain and to slow down the rate of reduction of joint space in patients suffering of OA. Contrary to these positive reports, data provided by two meta-analysis studies showed a minimal or nonexistent benefit from CS administration\cite{114,115}. However, many criticisms have been addressed to these studies, such as the huge heterogeneity between trials\cite{116} considering that treatment is known to vary in relation to disease severity, as confirmed by Sawitzke et al.\cite{117} and the high variability in the origin, potency and purity of CS samples used in the evaluated studies\cite{8,9}. Meta-analysis based on no evidence of heterogeneity across the trials showed positive effects of CS as a SYSADOA and S/DMOAD agent (Table 8).

The results of the all quoted clinical studies conducted on a huge number of patients and published in literature under the category of long-term stud-
eries (6 to 40 months) confirmed a total absence of toxicity of CS orally administered at doses of 1 to 2 g/day. Further clinical experiences confirmed the absence of major adverse reactions. Only minor adverse reactions have been reported in the course of these studies generally occurring at the beginning of the treatment and generally involving the gastrointestinal tract. A few cases of cutaneous rash were reported but their relation to the treatment could not be clearly demonstrated.
SAFETY CONCERNS OF ANIMAL-DERIVED CHONDROITIN SULFATE
CS, like other natural polysaccharides, is derived from animal sources by extraction and purification processes\cite{86}. As a consequence, source material, manufacturing processes, the presence of contaminants and many other factors contribute to the quality, structure and physico-chemical properties of the final product and of the overall biological and pharmacological activities of these agents. As above described, CS has a complex structure that is known to change with the tissue, organ and species\cite{86}. Furthermore, as well known, the biological and pharmacological properties may vary with the structure and the oral absorption may be influenced by the different physico-chemical properties\cite{95,96}. Finally, due to the extractive origin of CS, the presence of virus or prions cannot be excluded\cite{118}, as well as other various natural bioactive (macro)molecules present as contaminants in CS extracts in different amount\cite{8,119}, or voluntary adulteration by similar artificial compounds\cite{120}, or a restriction use related to religious issues (Figure 14).

**Figure 14.** Factors responsible for the heterogeneity of structure, properties and purity of animal-derived CS.
Animal-derived CS possess high variability and have different physical-chemical profile according to the various sources\cite{8,86,119}. Moreover, a mix of different animal tissues and sources are possible during extractive procedures producing a final product having varied characteristics and not well identified profile, influencing oral absorption and activity\cite{8,119}.

Quality of different CS preparations are also dependent by extraction and purification processes which may introduce further modifications of the structural characteristics and properties, such as depolymerization, desulfation and/or chemical modifications. The use of not controlled raw animal material (tissues, bones and cartilages but also soft organs) poses the problem of a final product having a highly variable structure with not well identified origin and poor reproducibility with the consequence of variable grade of purity, biological effects, presence of contaminants, clinical efficacy and safety\cite{8,9,119}.

These aspects pose a serious problem for the final consumer of the pharmaceutical or nutraceutical products that is related to the declaration on the label of the real origin of the active ingredient and to the label traceability of CS.
The industrial production of CS uses animal tissue sources as raw material, derived from different species of animals. Currently it generally relies on bovine, porcine, chicken or cartilaginous fish such as sharks and skate by-products, in particular cartilage, as raw material\cite{8,119}. More important, a mix of all these sources is possible producing a CS final product having mixed characteristics and not well identified activities.

Although all animal CS have an identical backbone, several studies indicate that the chemical composition (sulfation, overall charge density, nature of PGs) and quality of CS changes with age of the animal, tissue, organ and it is specific for each species (see Tables 4 and 9 for example).

Moreover, as a result of the biosynthetic processes related to specific tissues and species, CS with different grades of polymerization may be biosynthesized producing macromolecules having various molecular weight, type and grade of sulfation, and polydispersity (Table 9).

Animal extractive CS has been shown to present a high content of not well

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CS Bovine</th>
<th>CS Porcine</th>
<th>CS Chicken</th>
<th>CS Shark</th>
<th>CS Raja</th>
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</thead>
<tbody>
<tr>
<td>Molecular mass (kDa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
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<td>9.0±13.0</td>
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<tr>
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<td></td>
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<td></td>
</tr>
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<td>6.0</td>
<td>8.0</td>
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<td>nd</td>
<td>nd</td>
<td>15.0</td>
<td>13.0</td>
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<td>ΔDi-2,4dis</td>
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<td>3.00±4.00</td>
<td>0.45±0.70</td>
<td>1.00±1.40</td>
</tr>
</tbody>
</table>

Table 9. General chemical composition and properties of CS from various common sources.
identified proteins, up to 3-6%\cite{8,119,121,122} as well as nucleic acids and their fragments derived from chemical treatment reaching 1-2% \cite{8,119,121,122} and other GAGs extracted during its manufacture, in particular DS and hyaluronic acid \cite{123} and a high percentage of immunogenic keratan sulfate \cite{124-127} (Figure 15).

Due to these structural variations and in addition to the possible presence of specific oligosaccharide sequences as well as the purity of the preparations required for therapy applications or in nutraceuticals, CS may have different properties and effects\cite{8,119}. In fact, different and peculiar activities have been reported depending on the CS structure [for an exhaustive report on CS properties see reference 86]. Furthermore, CS is generally orally administered during the therapy as a nutraceutical supplement and different bioavailability and pharmacokinetic parameters have been reported to change depending on its structural characteristics and origin\cite{95-97}. 

**Figure 15.** Factors responsible for the heterogeneity of structure, properties and purity of animal-derived CS.
Finally, the use of animal-derived source for producing commercial CS represents a concern also for vegetarians and people with dietary restrictions related to religious and supply issues. Religious reasons and/or the practice of abstaining from the use of animal products have precluded the introduction of CS dietary supplements both in key emerging markets (Middle East and Asia) and in consolidated markets (North America and Europe).

### 2.2.1 NATURAL CONTAMINATIONS

Commercial CS, derived from animal sources, is obtained with long and complex procedures of extraction and purification with the aim to attempt elimination of the other (macro)molecules present as natural contaminants\[^86\]. At the same time, contamination of CS may derive from the same processes of extraction and purification.

#### 2.2.1.1 Natural polysaccharides

Depending on the purification protocols, CS extracts may have a variable grade of purity due to the presence of not desired side-products\[^119\].

Contamination by other polysaccharides such as other GAGs is possible along with the presence of nucleic acids, proteins and other (macro)molecules. Moreover, not controlled preparative processes may introduce important structural modifications on the CS backbone such as desulfation, depolymerization, oxidation/reduction and/or introduction of chemical modifications producing a polysaccharide having poor or no biological properties\[^8,119,125,126\].

In particular, DS with the presence of its building blocks made of iduronic acid and hyaluronic acid in various percentages in raw materials and formulations have been detected and declared in scientific papers\[^123\].

Moreover, keratan sulfate was detected in batches from shark cartilage, averaging 16% of the total GAGs\[^124,125\]. Its unexpected high percentage
compromises the desired amounts of the real ingredient specified on
the label claims and forewarns the pharmacopeias to update their mono-
graphs.

In addition, this finding also alerts the manufacturers for improved isolation
procedures as well as the supervisory agencies for better audits. Indeed,
kerratan sulfate, involved in the structure of cartilage proteoglycan aggrecan,
was detected in CS extracts and finished preparations of various origin, ob-
viously as CS is extracted from cartilage\textsuperscript{[126]}. A total of 15 samples, including
four samples of CS as laboratory reagents, one sample of CS as a food ad-
ditive and ten samples of dietary supplements containing CS were exam-
ined to detect keratan sulfate in these samples by using immunodiffusion
and enzyme-linked immunosorbent assay (ELISA) with anti-keratan sulfate
monoclonal antibody. With the exception of three samples of CS as labora-
atory reagents, all samples were found to contain varying amounts of keratan
sulfate. Finally, keratan sulfate possesses immunogenic capacities able to
develop immune reactions\textsuperscript{[127]}.

2.2.1.2 Virus or prions (or infective agents)
The animal origin poses potential consumer safety problems associated
with the possible presence of pathogen contamination and transmissible
infective agents (bacteria residues, viruses and prions) such as those caus-
ing spongiform encephalopathy in bovines (BSE), foot-and-mouth disease,
influenza spread in birds and other animal diseases\textsuperscript{[118]} (Figure 16).

The presence of prions may be easily demonstrated with a diagnostic test
and current available testing is very limited and expensive. Prions adhere
very tenaciously to surfaces making them hard to remove and can be re-
deposited on other surfaces during processing. Prions are also resistant
to protease treatment, certain chemical agents and heat denaturation and
represent a major concern for pharmaceutical products derived from ani-
mals. Other pathogens are also a concern because of similar attributes as
Prions for the potential risk of carry-over into a final purified product.
2.2.1.3 Natural polymers: proteins and nucleic acids
Animal CS extracts and derived final products for pharmaceutical and/or nutraceutical markets are also contaminated with (macro)molecules possessing similar properties of CS that are co-purified during CS extraction from tissues, such as in particular nucleic acids\textsuperscript{123} and proteins. In fact, animal CS is contaminated of a variable content of DNA and RNA depending on the purification protocols adopted and on the source\textsuperscript{[8,119].} Moreover, some of these proteins may have allergenic potential able to develop immune reactions (Figure 17).

Finally, we should consider that CS, like other natural sulfated GAGs, is covalently linked to a core protein to form the PGs. As a consequence, during the extractive procedures involving a proteolytic treatment, polypeptide sequences still remain linked to the CS backbone in a variable content\textsuperscript{[86].}
Figure 17. Presence of other (macro) molecules in animal-derived CS and their effects.
2.3 ADULTERATIONS

Contamination of animal-derived CS may also derive from the voluntary adulteration with cheaper polysaccharides and substances that cannot be determined with usual analytical controls utilized in the nutraceutical industry (Figure 18).

Chondroitin is now acknowledged within the nutraceuticals industry as one of the most adulterated supplements in the market, a problem which is widespread, deteriorating and on-going. Furthermore, the extent of adulteration is probably underestimated, with much of the adulteration going undetected, encouraged and abetted by the lack of industry-wide validation methods.

The recent application of specific analytical methods such as HPLC and electrophoresis on commercially available supplements has allowed to demonstrate not only the poor quality of these finished products in the global market, with a lower concentration of CS than specified in the label, but also the intentional adulteration of them [128-131].

Figure 18. Artificially (“FAKE”) adulterated animal-derived CS.
The adulteration is pursued with molecules which may influence non-specific wide-used methods of CS quantitation, such as the Cetylpyridinium Chloride (CPC) titration, to give an artificially inflated estimate of CS concentration\textsuperscript{132}.

The materials often used for adulteration may be carrageenan, proteins and surfactants, cheaper polysaccharides (Figure 19). Of the known chondroitin adulterants identified to date, including sodium alginate, propylene glycol alginate sulfate sodium, there is also the sodium hexametaphosphate, commonly used as a detergent or as a water-treatment additive and sold under the commercial name Calgon\textsuperscript{132}. These molecules can be separated from CS by their difference in electrophoretic mobility or other specific analytical approaches, but not with common largely used methods\textsuperscript{8,119,132} (Figure 20).

One industry trend which drives adulteration is price pressure. The majority (80-90\%) of the CS supplied to the US and Europe markets originates from China and intentional adulteration persists due to pricing pressure. This pushes the nutraceutical manufacturer to minimize ingredient costs by using substandard sourcing practices\textsuperscript{133}. This aspect related to price pressure is also related to minimize analytical controls just to common non-specific and inexpensive tests (Figure 20). As a consequence of this scenario, some raw
materials and dietary supplement products contain less than the claimed amount of CS and, in some cases, only 5-10%, which is a huge fraud to clients, traders and consumers.

Figure 20. Analytical procedures and related implications to check the adulterated animal-derived CS.
While pharmaceutical supply-chain requires very strict traceability to minimize (rather than eliminate) risks of contamination, animal-derived raw materials for nutraceutical market currently derives from not-well identified origin. The source of animals and their geographical origin, the nature of animal material used in manufacture and any procedures in place to avoid cross-contamination with higher risk materials, production process(es) including the quality assurance system in place to ensure product consistency and traceability, are not regulated and often not controlled. Moreover, seasonal, geographical and subspecies variations may alter the product obtained from a given animal species. Finally, some countries do not have a strict biosecurity system described as “implementation of practices that create barriers in order to reduce the risk of the introduction and spread of disease agents”.

Animal-derived CS suppliers have no regulatory requirements to provide materials with batch-to-batch reproducibility and free of contaminants. There are no rules to be transparent about their own processes and supply chains and to provide accurate information. Moreover, the missing guidelines allow manufacturers of finished products to change often the sources of supply of the CS raw materials for competitive advantages.

Manufacturers should ensure traceability of raw material across the supply chain until the finished product, with in-house analytical evaluation made of each lot supplied in order to guarantee same quality, reproducibility and safety, and avoid also misleading label information on finished products, as already described. The cost of such analytical controls and quality procedures would be so high (see Figure 20) that the dietary supplement manufacturers would not be competitive in the market price with loss of market competitiveness and, for this reason, not generally applied.

On the contrary, manufacturers often “qualify” brokers and suppliers by Self-Audit questionnaires and verification of Certificate of Analysis without testing raw materials or by using less expensive, but not consistent, analytical methods, with serious ineffectiveness to assess critical suppliers.
The industry-wide methods for analysis of CS are titration methods using CPC titration from United States Pharmacopeia (USP). Recent testing on CS dietary supplements has revealed several flaws in this older testing methods. The titration method has relevant limits because can be tricked by the use of expensive natural and/or artificial adulterants and cannot distinguish between CS and other material having similar physico-chemical properties, like nucleic acids, anionic proteins, other polysaccharides, etc.

Moreover, the origin of the CS utilized in a finished product can only be detected by rather expensive and detailed biochemical/structural analyses by determining the disaccharide/oligosaccharide pattern and molecular mass parameters\(^{[119]}\). As a consequence, today it is recognized the need to use a combination of specific analytical methods to define the final quality, origin and properties of the product. Such methods include chemical composition analysis, electrophoresis, HPSEC, immunochemical reactions, selective enzymatic digestion that further requires resolution using sophisticated techniques including HPLC, polyacrylamide-gel electrophoresis (PAGE), FACE, capillary electrophoresis, mass spectroscopy, and NMR spectroscopy\(^{[112,119]}\). CS profile is readily achieved using well-established laboratory techniques, particularly HPLC and electrophoresis. These techniques, applied after enzymatic digestion of CS into smaller components, can distinguish between CS derived from different animal or marine sources based on disaccharide content, patterns of sulfation and molecular size of the polymer. Anyway, a big problem still remains for the complete identification of the origin of CS when mixed sources material is used to its production or cross-contamination is possible.

The previous reported information has been confirmed in many studies in which CS finished products have been found of poor quality and not conformed to label declaration. In fact, previous studies reported a quantitative determination of CS in raw materials and various pharmaceutical preparations, and its content and properties in shark cartilage
powders used as nutraceutical supplements. Several low-quality CS raw materials were identified and the purity and origin of the preparations were found to be inconsistent with the specifications claimed on the product labels in several Countries[128-133].
The case study of heparin happened in February 2008 is well known. The China-derived heparin was contaminated by oversulfated CS having a very high charge density quite similar to natural heparin, derived from an unspecific over-sulfation, which provoked hundreds of deaths in Europe and USA when administered iv[134]. This terrible event related to intentional adulteration of an important natural drug derived from animals induced a strong increase in research for the production of a possible bioengineered heparin[135]. As for heparin, intentional adulteration of CS is difficult to detect since the tissue supply chain for CS in slaughterhouses generally lacks current good manufacturing processes (cGMP) oversight.

Furthermore, CS is derived from a variety of animal tissues and these animal source materials can contain infectious agents leading to the potential transmission of viral and prion diseases. This is more important by considering that the mixing of CS obtained from different animal species is highly suspected. Even if specific and high-level analytical methods are generally able to distinguish the various sources, the detection of the presence of small amounts of different raw material source still remains very difficult and a sure challenge.

Moreover, the susceptibility of animal populations to infectious disease, such as porcine epidemics in China, overharvesting, or environmental concerns can dramatically reduce the supply animals for CS production. Additionally, product quality can vary with environmental factors and animal subspecies, providing additional difficulties for CS quality and properties standardization.

Finally, commercial suppliers must answer to the increased worldwide demand particularly in developing countries. As a consequence, the urgent solution to this scenario is the introduction and utilization in the current market of the non-animal derived source CS Mythocondro®.
MYTHOCONDRO®:
THE CHONDROITIN SULFATE
OF NON-ANIMAL ORIGIN
3.1 STATE OF THE ART

The potential consumer safety and quality problems previously discussed associated with the use of animal-derived CS, suitable for pharmaceutical and nutraceutical applications, have historically prompted the search for an alternative source.

An innovative patented production process has been developed in the last years by Gnosis, an Italian-based biotechnology company, leading to an innovative product closely resembling natural CS, with physico-chemical, structural and functional properties comparable to those of animal-derived products but without any concern related to origin of the production, the possible presence of transmissible infective agents, the contamination and adulterations typical of animal-derived raw materials.

The new ingredient, branded as Mythocondro®, is the first CS obtained with a fermentation-based manufacturing process followed by selective sulfation. Mythocondro® warrants high purity, clear identity profile, batch-to-batch reproducibility, established safety with an extremely low content of proteins and other (macro)molecules, as well as a superior biological activity.

Mythocondro® is the result of several years of research and development and has generated five international patents based upon scientific advances in the biotechnological areas.

Mythocondro® has been extensively studied in order to evaluate its characteristics, safety profile and efficacy. Pre-clinical and clinical studies have been carried out demonstrating superior biological activity versus animal-derived CS both in vitro and in vivo.
Specific bacterial strains produce capsular polysaccharides showing a strong homology with chondroitin. The study of the DNA region coding for the enzymes responsible for the biosynthesis of the polysaccharide has been deeply detailed, leading to the selection of non-GMO *E. coli* strains producing high levels of the polysaccharide and low levels of impurities. The selected bacterial strain produces a surface capsular polysaccharide named K4, that is identical to unsulfated chondroitin except for carrying fructose residues in the C3 position of the glucuronic acid residues.

The development of selective growing conditions of this bacterial strains able to result in high levels of polysaccharide was a big challenge for Gnosis, by also considering the goal to use a non-animal origin medium free from the potential variations in the quality of natural medium ingredients.

After the fermentation, the polysaccharide produced in high yields is purified, and hydrolyzed under specific conditions to remove the fructose residues. The recovered chondroitin-like polysaccharide undergoes regioselective sulfation, modulated in order to obtain a CS (Figure 21) which is mainly monosulfated thus avoiding the formation of polysulfated disaccharides which are common in the majority of chemical sulfation processes conferring limited biological activity and safety concerns.

The result of this innovative approach is a non-animal CS with homogeneous structure and the presence of sulfate groups in defined positions, a

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**Figure 21.** The production of Mythocondro®.
constant charge density and molecular mass parameters overall largely sim-
ilar and comparable to animal-derived samples eliminating the safety con-
cerns raised by previously described animal-derived.

The non-extractive origin guarantees:

| • Homogeneous structure and physico-chemical properties |
|                                                    |
| • High purity                                      |
|                                                    |
| • Very low content of proteins                     |
|                                                    |
| • No presence of virus and/or prions               |
|                                                    |
| • Highly and point-by-point controlled production process for highly controlled and reproducible final product |
3.3 MYTHOCONDRO® STRUCTURAL FEATURES AND PHYSICAL-CHEMICAL PROPERTIES

Mythocondro® has homogeneous structure and physico-chemical properties in particular for the presence of sulfate groups in defined positions, a constant charge density and molecular mass parameters. Moreover, compared to all known animal-derived CS, Mythocondro® is characterized by an extremely low content of natural contaminants, i.e. proteins, nucleic acids, other polysaccharides, largely reported to be present in extractive CS strictly due to its chemical nature and purification procedures adopted, as previously largely discussed. As a consequence, Mythocondro® possesses very high purity level.

Specific and sensitive analytical procedures identify the structure of non-animal CS and characterize its properties and purity.

Molecular mass parameters and profile is determined by HPSEC (high-performance size-exclusion chromatography) and further by PAGE that confirmed a medium molecular mass value lower than about 10 kDa, a homogeneous profile and low polydispersity (Figure 22). In fact, the polydispersivity in the molecular mass of Mythocondro®, an important factor for biological actions, is maintained due to the polydisperse nature of the polymer K4 which undergoes hydrolysis and regiosulfation in the manufacturing process.

![Figure 22. HPSEC and PAGE characterization of Mythocondro®.](image)
Various kinds of electrophoresis, agarose-gel according to EP (European Pharmacopeia) and acetate of cellulose (ACE) according to USP show a profile with no other possible natural polysaccharide present at LOD (Limit Of Detection) lower than 0.2% (Figure 23). Moreover, enzymatic HPLC (eHPLC according to AOAC International), 1H-NMR and 13C-NMR defined the structural characteristics of Mythocondro® (Figure 23).

Currently, USP and EP allow up to 6% and 3% of undefined protein content respectively in their specification while Mythocondro® has lower than 0.1% of well characterized proteins. Being this parameter possibly linked to allergies onset, Mythocondro® presents an extremely diminished risk compared to animal-derived CS. In fact, depending on the extractive and purification procedures adopted to produce CS from animal tissues (and we should keep in mind that more purified is the CS and more purification steps are required and more time-and money-expensive is the final product), up to 6% proteins may be present. On the contrary, Mythocondro® has been found to contain about <0.1% proteins due to its specific nature and production.

**Figure 23.** Specific and sensitive analytical procedures characterize the structure of Mythocondro®.
3.4 BIOLOGICAL ACTIVITY OF MYTHOCONDRO®

3.4.1 Toxicological studies

By using established procedures, *E. coli* strain was evaluated for any toxicogenic potentials. None of the genes searched for was found to be present in the total DNA extracted from used strain, showing that the strain in question is genetically unable to produce virulence factors and toxins. Moreover, before removal from the fermenter, the culture is always inactivated by heating.

Furthermore, Mythocondro® has been positively tested in a validated Safety Assessment Study published on Food and Chemical Toxicology\textsuperscript{[136]}. The objective of this study, performed according to OECD (Organization for Economic Co-operation and Development) guidelines - in use for pharmaceutical products - was to investigate:

- the long-term repeat dose toxicity (subchronic toxicity study) of the Mythocondro®, when administered daily for 90-days via oral gavage to Sprague Dawley rats;

- the potential genotoxic effects of Mythocondro®, investigated in bacterial reverse mutation assay (Ames test) using *Salmonella typhimurium* strains, *in vitro* chromosomal aberration assay in CHO and *in vitro* mutation in mouse lymphoma cells;

- the pharmacokinetics of Mythocondro®, in a comparative pharmacokinetic single dose, open label, randomized, two-way cross-over study in 24 human subjects with known bovine CS.

The study fully validates the excellent safety profile of the fermentation-based CS Mythocondro® not revealing any mutagenicity, genotoxicity and with a no-observed-adverse-effect level (NOAEL) of 1000 mg/kg (NOAEL of 1000 mg/kg bw/day). The available evidence suggests that recommended daily intake of microbial derived Mythocondro®, is very safe.
3.4.2 Permeability

One of the major problems associated with oral administration of native animal CS is the low bioavailability. Intestinal animal high molecular weight CS absorption amounts to about 1-5%\[95,96\].

*In vitro* and *in vivo* clinical studies report that CS is generally hydrolyzed to smaller oligosaccharides by means of specific enzymes produced by the intestinal flora\[98,137\]. By considering the oligosaccharide fraction, the total intestinal CS uptake amounts to about 20-23%\[138\]. This documented low bioavailability has limited the CS efficacy as a dietary supplement so far. Molecular weight and the range of polydispersity may impact on the pharmacokinetics profile of supplemented CS.

Mythocondro® shows an improved intestinal epithelial permeability compared to animal CS samples due to its lower molecular mass, without the need of further hydrolysis by the intestinal flora.

The variation in molecular masses and sulfation patterns make CS a heterogeneous molecule. Several studies are available and hypotheses have been made regarding differences in the bioavailability of CS due to its structural variations\[92,93,95,96,107,139,140\].

Mechanisms of transportation across the intestinal barrier include transcellular transportation (carrier mediated or active transportation, endocytosis and pinocytosis), paracellular transportation or passive diffusion through the tight junctions. Mode of transportation depends on the physicochemical properties of the compound such as structure, molecular weight or size, charge distribution and hydrophobicity\[141\]. As identified in the report\[141\] and recommended by European Center for the Validation of Alternative Methods (ECVAM), examples of *in vitro* models of intestinal absorption include the human adenocarcinoma cell line Caco-2. Caco-2 cells are isolated from a primary human colonic tumor. When cultured under standard cell culture conditions, they spontaneously differentiate into polarized enterocytes characterized by apical
and basolateral sides, by the presence of tight junctions and the expression of most of the small intestinal brush border enzymes and transporters.

Mythocondro® showed a better permeability in the Caco-2 cells model when compared to CS samples of various animal sources, being its permeability coefficient up to 65 times higher (Figure 24).

![Figure 24. Coefficients of effective permeability (Peff) for CS samples. Mythocondro® is significantly higher than the rest of the samples.](image)

### 3.4.3 Bioavailability

Pharmacokinetic profile of Mythocondro® versus a sample of bovine extracted CS has been evaluated in healthy volunteers. In this single center, single dose, open-label, randomized, two-way, cross-over study, 24 male and female subjects [18-65 years; BMI- 23.67 ± 2.52-] received both test and reference treatment and completed the study as per protocol. Single doses of 2400 mg of test or reference product were administered under fasting conditions in two consecutive study periods according to the randomized cross-over design.

The trial and the methodologies for statistical comparison between treatments
were designed taking into account the recommendations of the Guideline on the investigation of bioequivalence (CPMP/QWP/EWP/1401/98 Rev. 1, January 2010). In pharmacokinetic study in humans, non-animal CS shows higher absorption as compared to CS of animal origin.

Mythocondro® showed an increased AUC0-24h compared to bovine CS already after 1 h from products administration, and this difference increased up to 24 h. This behavior is evident in Figure 25 where a higher plasmatic concentration of Mythocondro® is showed and it is evident its capacity to remain higher for a long time in human plasma for more than 24 h if compared to bovine CS.

In particular, 5 h after administration, the plasmatic concentration of Mythocondro® remains up to 89% higher than bovine CS (Figure 25). Finally, the quite similar value of $C_{\text{max}}$ for both formulations further supports data illustrated in Figure 25 and demonstrates that the different plasmatic behavior is

![Figure 25. Plasma CS concentration (mg/mL) vs. time profiles following administration of test and reference formulations to human subjects.](image-url)
strictly related to the capacity of Mythocondro® to remain for a longer time in the blood compartment with a higher concentration compared to bovine CS.

The human clinical trial has not only produced data on the safety, but allows to claim a higher bioavailability of Mythocondro® versus bovine CS of 43%.

3.4.4 Anti-arthritic effect

Adjuvant induced Arthritis (AA) in rats is an animal model of polyarthritis which allows to monitor the disease processes in the acute phase (days 14-21) and in the subchronic phase (day 28) of the disease. The advantages of this model are its many similarities with RA and polyarthritis diseases in man, such as symmetrical joint involvement, persistent joint inflammation, synovial hyperplasia and a good response to most therapies effective in RA[142].

AA in rats is widely accepted and considered by the scientific community as a good experimental and preclinical model to test arthritis symptoms and their treatments. AA was induced by a single intradermal injection of *Mycobacterium butyricum* in incomplete Freund’s adjuvant, a solution of antigen emulsified in mineral oil and used as an immuno-potentiator (booster).

The anti-inflammatory effect of Mythocondro® (Low-Molecular-Mass CS: LMM-CS) was assessed through the reduction of arthritic score as well as the evaluation of plasmatic levels of pro-inflammatory cytokines IL-1β and IL-6 as well as by γ-glutamyltransferase (GGT) activity in hind paw joint tissue homogenates and plasmatic C-reactive protein (CRP). Arthritic score is a complex index of pathology progression assembling several clinical parameters including periarticular erythema, edema and the local inflammation at the site of injection. The study included healthy animals, untreated arthritic animals and arthritic animals who received 900 mg/kg daily of bovine CS, a high-molecular mass biotechnological CS (HMM-CS) and Mythocondro® (LMM-CS).
Mythocondro® significantly reduced the arthritic score by up to about 30% from 14 to 28 days (Figure 26). In contrast, no significant differences were observed for HMM-CS, even if a trend in its capacity to decrease the arthritic score by up to about 11% was observed.

Additionally, the effect was confirmed by the reduced production of pro-inflammatory cytokines, GGT and CRP in plasma (Figure 27). Mythocondro® was able to significantly decrease GGT activity by approximately 31% and plasmatic CRP levels by about 9%. Both non-animal CS samples were effective in reducing plasmatic levels of pro-inflammatory cytokines. A greater efficacy was also observed for Mythocondro® compared with the pharmaceutical-grade CS of bovine origin, while the efficacy of the HMM-CS sample was found to be rather similar. The greater effect of Mythocondro® in reducing arthritic parameters may be related to its lower molecular mass and increased bioavailability with respect to HMM-CS and animal CS.

Figure 26. Arthritic score of a pharmaceutical-grade animal CS, a HMM-CS and Mythocondro® (LMM-CS) in the adjuvant arthritis animal model.
Mythocondro® showed to be effective to slow down arthritis development and to reduce disease markers after 28 days of treatment supporting further clinical phase in humans. This preclinical study was published in 2014 by Bauerova et al. [143].

3.4.5 Why Mythocondro® is better than animal chondroitin sulfate?

Some key points illustrated in the following last Table may be considered to compare animal CS, the actual state of art, with Mythocondro®, opening a new era of CS.
## THE EVOLUTION OF CHONDROITIN SULFATE

<table>
<thead>
<tr>
<th>Mythocondro®</th>
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</tr>
<tr>
<td><strong>Low molecular weight: 5 - 12 KDa</strong></td>
<td>High Molecular weight</td>
</tr>
<tr>
<td><strong>A controlled, reliable and reproducible source of CS with high purity</strong></td>
<td>Not strictly regulated</td>
</tr>
<tr>
<td></td>
<td>✗ Not well-identified origin</td>
</tr>
<tr>
<td></td>
<td>✗ Highly variable structure</td>
</tr>
<tr>
<td></td>
<td>✗ Poor batch-to-batch reproducibility</td>
</tr>
<tr>
<td><strong>A very low protein content (less than 0.5% d.b.). It is usually 0.1% d.b. No presence of other polymers or nucleic acids</strong></td>
<td>High protein level (up to 3% (EP), and up to 6% (USP) d.b.). Not well identified proteins and nucleic acids and their fragments derived from chemical treatment contamination potentially responsible for allergic reactions in the consumers. Presence of immunogenic keratan sulfate</td>
</tr>
<tr>
<td><strong>Highly and point-by-point controlled process of production for a highly purified and reproducible final product</strong></td>
<td>Process of extraction generally not controlled causing possible modifications of the CS structure, such as desulfatation, depolymerisation, oxidation, etc</td>
</tr>
<tr>
<td><strong>A substantial absence of unusually sulfated and over-sulfated disaccharides. Mythocondro® has a very precise sulfation pattern</strong></td>
<td>Standard animal CS origin is made up of different CS (mainly C4S sulfated in position 4 of GalNAc, C6S sulfated in position 6 of GalNAc but also variable % of C0S, non-sulfated disaccharide. Variable % of disulfated (and trisulfated) disaccharides</td>
</tr>
<tr>
<td><strong>Clinically proven superior bioavailability of Mythocondro® than animal CS due to lower molecular weight and an improved intestinal epithelial permeability</strong></td>
<td>Lesser bioavailability</td>
</tr>
<tr>
<td><strong>Proved anti-inflammatory and protective properties in a preclinical arthritis animal model</strong></td>
<td>In general, no evaluation of any biological activity</td>
</tr>
<tr>
<td><strong>Mythocondro® is totally safe: no toxicity in acute and chronic animal models. No prions or animal viruses can be present due to the fermentation origin of the product</strong></td>
<td>The animal origin poses a potential consumer safety problem associated with the possible presence of transmissible infective agents (bacteria, viruses and prions)</td>
</tr>
<tr>
<td><strong>Mythocondro® is suitable for vegetarians and all religions as it is of natural origin</strong></td>
<td>Some animal origins are not suitable neither for vegetarians nor for consumers of different religions</td>
</tr>
</tbody>
</table>


76. Inoue K, Hukuda S, Fardellon P et al. Prevalence of large-joint osteoarthritis in


TH E EV OLUT IO N O F C HO NDRO IT I N SULF AT E


THE EVOLUTION OF CHONDROITIN SULFATE


GLOSSARY

AA=adjuvant induced arthritis
ACE=acetate of cellulose
BMI=body mass index
BSE=bovine spongiform encephalopathy
cGMP=current good manufacturing processes
CPC=cetylpyridinium chloride
CRP=C-reactive protein
CS=chondroitin sulfate
CSA=chondroitin-4-sulfate
CSC=chondroitin-6-sulfate
DMOAD=disease modifying potential of CS
DS=dermatan sulfate
ECM=extracellular matrix
ECVAM=European center for the validation of alternative methods
ELISA=enzyme-linked immunosorbent assay
EP=European Pharmacopoeia
EULAR=European League Against Rheumatism
GAG=glycosaminoglycan
GGT=gamma-glutamyltransferase
GlcN=glucosamine
HMM-CS=high-molecular mass biotechnological CS
HPSEC=high-performance size-exclusion chromatography
IL=interleukins
LMM-CS=low-molecular-mass-CS
LOD=limit of detection
MMPs=metalloproteinases
NF-kB=nuclear factor-kB
NO=nitric oxide
NOAEL=no-observed-adverse-effect level
OA=osteoarthritis
OARSI=osteoarthritis research society international
OECD=Organization for Economic Co-operation and Development
PAGE=polyacrylamide-gel electrophoresis
Peff=effective permeability
PGs=proteoglycans
RA=rheumatoid arthritis
S/DMOAD=structure/disease modifying anti-osteoarthritis drug
SYSADOAs=symptomatic slow-acting drugs for osteoarthritis
USP=United States Pharmacopeia
WHO=World Health Organization
Nicola Volpi is Associate Professor of Biochemistry at the University of Modena and Reggio Emilia, Italy. He has worked in the field of complex macromolecules, i.e. polysaccharides, glycosaminoglycans, glycoproteins and proteoglycans since 1990, for over 25 years.

Prof. Volpi is one of the worldwide expert in the field of chondroitin sulfate and his studies and researches over the last 25 years have made a great impact on the international scientific community as documented by his many publications, invitations to several national and international congresses in addition to three published international books as Editor. He developed several preparatory and analytical techniques for the study of the structure and properties of this complex biomolecule. His skills in the application of the analytical methodologies essential for the understanding of the structure, properties and quality of chondroitin sulfate and derivatives is documented by his publications of high international level and patents.

Prof. Volpi is currently collaborating with Italian and International research groups with a view to developing new methodologies and applying these approaches to new molecular studies. Furthermore, he also collaborates with Companies to develop (macro)molecules having new improved biological and potential pharmacological properties.

Prof. Volpi has been member of the USP (U.S. Pharmacopoeia) Commission in 2000 for “Analytical determination of Chondroitin sulfate sodium”.