

## Immunomodulatory and anti-inflammatory effects of chondroitin sulphate

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- Biochemistry of chondroitin sulphate
- Mechanism of action of chondroitin sulphate
  - Effect of chondroitin sulphate on the chondrocyte
  - Effect of chondroitin sulphate on the synovial membrane
  - Effect of chondroitin sulphate on subchondral bone
- Human use of chondroitin sulphate
  - Chondroitin sulphate in osteoarthritis
  - Chondroitin sulphate in psoriasis
  - Chondroitin sulphate in atherosclerosis
  - Chondroitin sulphate in IBD
  - Chondroitin sulphate in degenerative diseases of the central nervous system (CNS)
  - Other autoimmune diseases that may benefit from chondroitin sulphate
- Conclusions

### Abstract

Chondroitin sulphate (CS) is a natural glycosaminoglycan present in the extracellular matrix and is formed by the 1–3 linkage of D-glucuronic acid to N-acetylgalactosamine. In chondrocytes, CS diminishes interleukin-1 $\beta$  (IL-1 $\beta$ )-induced increases in p38 mitogen-activated protein kinase (p38MAPK) and signal-regulated kinase 1/2 (Erk1/2) phosphorylation, and decreases nuclear factor- $\kappa$ B (NF- $\kappa$ B) nuclear translocation and as a consequence, reduces the formation of pro-inflammatory cytokines, IL-1 $\beta$  and TNF- $\alpha$ , and pro-inflammatory enzymes, such as phospholipase A2 (PLA2), cyclooxygenase 2 (COX-2) and nitric oxide synthase-2 (NOS-2). The mechanism of action of CS explains its beneficial effect on the cartilage, synovial membrane and subchondral bone. On the other hand, *in vivo*, CS given orally prevents hepatic NF- $\kappa$ B nuclear translocation, suggesting that systemic CS may elicit an anti-inflammatory effect in many tissues besides the articulation. 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. The review of the literature suggest 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.

**Keywords:** chondroitin sulphate • inflammation • autoimmune diseases • psoriasis • atherosclerosis • inflammatory bowel disease • Alzheimer's disease • Parkinson disease • multiple sclerosis

### Biochemistry of chondroitin sulphate

Chondroitin sulphate (CS) is a natural glycosaminoglycan (GAG) present in the extracellular matrix surrounding cells, especially in the cartilage, skin, blood vessels, ligaments and tendons, where it forms an essential component of proteoglycans (PG) [1, 2]. CS is the main disaccharide unit of cartilage GAG, formed by the 1–3

linkage of D-glucuronic acid to N-acetylgalactosamine. The disaccharide units are attached by  $\beta$  1–4 galactosamine links. The galactosamine residues are sulphated either in position 4 ( $\Delta$ di-4S), 6 ( $\Delta$ di-6S) or 4 and 6 ( $\Delta$ di-4,6S). The sulphate groups along with the carboxyl groups of glucuronic acid are ionized, conferring a

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negative charge. In the extracellular matrix of the cartilage, about 100 chains of CS, each containing 50 to 60 disaccharide units, are covalently attached to a long polypeptide backbone composed of more than 2000 amino acids. The polypeptide with the CS chains is O-linked to hyaluronic acid forming the PG. The domain of the core protein located between the link protein and the CS region is occupied by keratan sulphate chains. The keratan sulphate disaccharide unit is formed by galactosido-1,4-N-acetylglucosamine-6-sulphate. Because of their high degree of hydration, CS-containing PG of articular cartilage are responsible for the viscoelastic properties of the tissue [3, 4].

## Mechanism of action of chondroitin sulphate

Because CS elicits a key role in the articulation, many research groups have focused on the role of CS on the chondrocytes, the synovial membrane and the subchondral bone.

### Effect of chondroitin sulphate on the chondrocyte

Repeated trauma on an articulation increases the release of cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), cytokines that play a key role in the development of osteoarthritis (OA). In chondrocytes, IL-1 $\beta$  activates extracellular signal-regulated kinase 1/2 (Erk1/2) and p38 mitogen-activated protein kinase (p38MAPK), and therefore induces the nuclear translocation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) and the activator protein-1 (AP-1). These transcription factors bind to consensus sequences of numerous pro-inflammatory genes, and initiate as well as maintain the inflammatory reaction in chondrocytes [5–7]. As a result, IL-1 $\beta$  increases the expression of matrix metalloproteinase-3 (MMP-3) [8], MMP-9 [9], MMP-13 [8, 10, 11], phospholipase A2 (PLA2) and cyclooxygenase 2 (COX-2) [11, 12], nitric oxide synthase-2 (NOS2), IL-1 $\beta$  and TNF- $\alpha$  [13].

Using chondrocytes stimulated by IL-1 $\beta$  as experimental model, it was demonstrated that CS diminishes IL-1 $\beta$ -induced NF- $\kappa$ B nuclear translocation and sodium nitroprusside-induced chondrocyte apoptosis. The effects of CS are mediated by inhibition of p38MAPK and Erk1/2 phosphorylation. These data suggest that the anti-inflammatory activity of CS is associated with the reduction of Erk1/2 and p38MAPK phosphorylation and nuclear translocation of NF- $\kappa$ B [14].

### Effect of chondroitin sulphate on the synovial membrane

Synovial tissue from patients with early osteoarthritis shows activated fibroblast-like synoviocyte (FLS), macrophages, T lympho-

cytes and mast cells infiltration [15]. Synovial FLS release IL-1 $\beta$ , IL-6, IL-8, MMP-1, MMP-2, MMP-3, MMP-13, MMP-14, MMP-16, tissue inhibitor of metalloproteinases-1 (TIMP-1), receptor activator of nuclear factor-kappa B ligand (RANKL), transforming growth factor- $\beta$  (TGF- $\beta$ ), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [16]. Activation of NF- $\kappa$ B increases FLS proliferation, and changes the phenotype of these cells into highly invasive FLS with great motility and ability to secrete cytokines and MMP-13 [17].

Inhibition of the I $\kappa$ B kinase (IKK) complex impedes the phosphorylation of the inhibitor of  $\kappa$ B (I $\kappa$ B $\alpha$ ) and as a consequence, prevents NF- $\kappa$ B activation. In synovial macrophages, inhibition of IKK diminishes IL-1 $\beta$ -induced production of IL-6; moreover, in rats with adjuvant-induced arthritis, intra-articular injection of a specific IKK- $\beta$  inhibitor reduces arthritis activity and bone destruction; synovial inflammation is also decreased as documented by the reduction in synovial cellularity, TNF- $\alpha$ , IL-1 $\beta$  concentrations and reduction of the volume of the paw [18]. The crucial role of NF- $\kappa$ B in the initiation of synovitis is further supported by the fact that the injection of a dominant-negative form of IKK- $\beta$  in the articulation of the rat with adjuvant-induced arthritis reduces synovial cellularity by 50%, and diminishes synovial concentrations of IL-1 $\beta$ , TNF- $\alpha$  and MMP-3 [19]. These results provide evidence that activation and nuclear translocation of NF- $\kappa$ B is an important step in the development of synovitis [20].

In patients with knee OA, CS diminishes the number of patients with signs of synovitis from 90 of 307 at baseline to 38 ( $P = 0.01$ ) at the end of 24 weeks of treatment [21]. This confirms, in human beings, observations reported in DBA/1J mice with a type II collagen-induced arthritis and treated for 9 weeks with various dosages of CS; infiltration of inflammatory cells, granulated tissue formation and proliferation of synovial lining cells were partially prevented by treatment with 1000 mg/kg/day of CS for 63 days [22].

The mechanism of action underlying the reduction of synovitis signs by CS remains poorly known. There is evidence that the inflammatory response in OA is closely associated with the activation and nuclear translocation of NF- $\kappa$ B, a phenomenon dependent upon the activation of p38MAPK, Erk1/2 and c-Jun N-terminal kinase (JNK) [23, 24]. Moreover, proliferation, motility, release of cytokines and matrix-degrading activity of FLS are associated with the activation and nuclear translocation of NF- $\kappa$ B [15, 17, 25].

Because in chondrocytes, CS diminishes Erk1/2 and p38MAPK and reduces IL-1 $\beta$ -induced NF- $\kappa$ B nuclear translocation [14], we might speculate that CS reduces NF- $\kappa$ B nuclear translocation in synovial cells, and so diminishes the synovial inflammatory reaction. Supporting such hypothesis, in human synoviocytes stimulated by IL-1 $\beta$ , the CS disaccharide ( $\Delta$ di-6S) reduces the nuclear translocation of NF- $\kappa$ B by 65% [26]. This observation is in agreement with the effect of  $\Delta$ di-4S and  $\Delta$ di-6S disaccharides in chondrocytes, for example they reduce NF- $\kappa$ B nuclear translocation [20]. Oral CS increases plasma concentrations of  $\Delta$ di-4S and  $\Delta$ di-6S [27], it is therefore conceivable that in human beings the decline of synovitis signs produced by CS may be explained, at

least in part, by the reduction in NF-κB nuclear translocation in synoviocytes and macrophages produced by CS disaccharides.

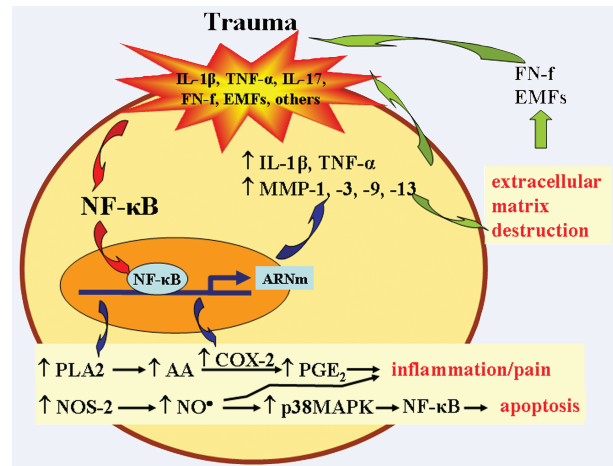
### Effect of chondroitin sulphate on subchondral bone

Recent evidence shows that altered osteoblast metabolism plays an important role in subchondral bone alterations, which in turn have been implicated in the progression and/or initiation of OA [28]. In human subchondral bone osteoblasts, CS up-regulates osteoprotegerin (OPG) expression and decreases RANKL expression [29]; as a consequence, CS increases the ratio of OPG/RANKL. Because the expression of RANKL is increased in abnormal osteoblasts with bone destruction [30], CS could exert a positive effect that may result in the reduction of the resorptive activity in subchondral bone. The mechanism of action underlying the effect of CS might be associated with the fact that induction of RANKL expression requires the activation of Erk1/2 and PI-3K/AKT pathways [31].

### Human use of chondroitin sulphate

The rationale for using CS as a treatment for OA was the decrease of CS in ageing patients with OA. In the late 1960s, anecdotal reports [32] suggested that CS disaccharides were reduced in synovial fluid of patients with OA, observations that were confirmed three decades later [33].

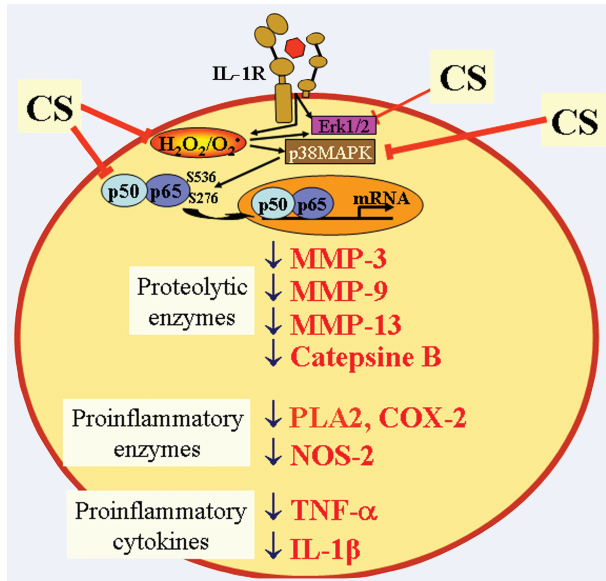
Osteoarthritis affects a majority of individuals over 60 years of age, and is characterized by focal areas of loss of articular cartilage, with varying degrees of osteophyte formation, subchondral bone change and synovitis, with local inflammation, pain and functional disability [34]. The pathophysiology of OA remains controversial. It has been proposed that the use of the joint implies multiple micro-trauma to the articular cartilage with the formation of extracellular matrix fragments (EMFs) and fibronectin (FN-f) (Fig. 1). These fragments bind to the integrin family of cell-surface receptors of the chondrocyte and promote the expression of MMPs, primarily MMP-13, aimed to cleave the EMFs [35–37]. The increase in expression of MMPs is accompanied by an enhanced synthesis of pro-inflammatory cytokines, essentially IL-1β and TNF-α, which will sustain the activation of chondrocytes and, moreover, will promote the formation of MMPs, aggrecanase, reactive oxygen intermediates, nitric oxide and lipid-derivative inflammatory mediators such as prostaglandins and leukotrienes. These mediators will enhance the catabolic activity of the chondrocyte, causing further destruction of the cartilage matrix. On the other hand, EMFs, IL-1β and TNF-α released into the synovial fluid activate macrophages and mastocytes in the synovial membrane originating the synovitis. Activation of synovial cells will result in a further release of IL-1β, TNF-α and MMPs that will contribute to the destruction of the cartilage matrix [16, 34, 38–40].



**Fig. 1** Multiple microtrauma to the articulation induce the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-17), extracellular matrix fragments (EMFs) and fibronectin fragments (FN-f) that bind to membrane receptors of the chondrocyte and activate NF-κB, and therefore promote the expression of matrix metalloproteases (MMPs), which further generate EMFs. Nuclear translocation of NF-κB will also increase the expression of phospholipase A2 (PLA2) and cyclooxygenase-2 (COX-2), enzymes that will release arachidonic acid and form prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) responsible for the inflammation and pain. NF-κB increases the expression of nitric oxide synthase-2 (NOS-2) and the production of nitric oxide (NO<sup>•</sup>) that will contribute to the inflammatory reaction, pain and apoptosis.

Cytokines, FN-f and EMFs by binding to membrane receptors activate signal transduction pathways, such as Erk1/2 and p38MAPK, which induce the nuclear translocation of NF-κB [39]. In the nucleus, NF-κB binds to the promoter of numerous genes and increases the transcription and expression of IL-1β, TNF-α, COX-2, NOS-2, PLA2 and MMP-1, -3, -9 and -13. Released MMPs further contribute to destroy the extracellular matrix and form additional FN-f and EMFs that, together with the cytokines, will perpetrate the inflammatory reaction of the chondrocytes and the synovial membrane. On the other hand, PLA2 will release arachidonic acid (AA) to generate prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) by COX-2, cause of inflammation and pain. The excessive production of nitric oxide contributes to increase the inflammatory reaction and pain [34, 40].

As outlined, cytokines have a crucial role in the onset and progression of OA [41]. For instance, IL-1β and TNF-α are implicated in the early development of arthritis, and IL-1β contributes to sustain the inflammatory reaction in later stages; IL-17 and IL-18 are also pro-inflammatory cytokines in the joint. Other cytokines released in the osteoarthritic joint have a regulatory role of the inflammation (IL-6, IL-8), or an inhibitory or anti-inflammatory function (IL-4, IL-10, IL-11, IL-13, IFN-γ and IL-1 receptor antagonist), or even an anabolic role, such as insulin-like growth factor 1 (IGF-1), transforming growth factor β (TGF-β), fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) [42, 43].



**Fig. 2** Chondroitin sulphate (CS) by inhibiting reactive oxygen species ( $H_2O_2$  or  $O_2^*$ ) and/or the extracellular signal-regulated kinase 1/2 (Erk1/2) and p38 mitogen-activated protein kinase (p38MAPK), reduces the nuclear translocation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B or p50/p65 heterodimer), and therefore CS diminishes the synthesis of proteolytic enzymes, such as the matrix metalloproteases (MMP-3, -9, -13) and cathepsin B, of pro-inflammatory enzymes, for example fosfolipase A2 (PLA2), cyclooxygenase-2 (COX-2) and nitric oxide synthase-2 (NOS-2), and of pro-inflammatory cytokines, for example TNF- $\alpha$  and IL-1 $\beta$ .

## Chondroitin sulphate in osteoarthritis

Randomized placebo-controlled clinical trials have demonstrated that CS reduces pain and improves articular function [44–46], reduces joint swelling and effusion [21] and prevents joint space narrowing of the knee [44, 46] and fingers [47, 48] more effectively than placebo. Accordingly, CS has been classified as a symptomatic slow-acting drug in osteoarthritis (SYSADOA) and a structure/disease-modifying anti-osteoarthritis drug (S/DMOAD) [44, 47].

The beneficial effects of CS in patients with OA result from different effects of CS on articular tissues, primarily the result of the immunomodulatory effect of CS, for example reduction of NF- $\kappa$ B nuclear translocation, decrease in the production of pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  and reduction in the expression and activity of NOS-2 and COX-2 (Fig. 2). Other effects of CS may contribute to its beneficial effect, such as the increase in the synthesis of articular cartilage PG, the reduction in the apoptosis of chondrocytes and the reduction of the synthesis and/or activity of MMPs [28, 49, 50].

Following its nuclear translocation, NF- $\kappa$ B enhances the transcription activity of a variety of genes encoding chemokines, cytokines, adhesion molecules, inflammatory-associated enzymes

and inhibitors to apoptosis [51]. Moreover, NF- $\kappa$ B signalling has been shown to be involved in lymphopoiesis and in the differentiation and activation of macrophages, osteoclasts, dendritic cells and granulocytes [52]. The NF- $\kappa$ B signalling pathway is closely linked to the regulation of inflammatory responses and survival of immune cells and accordingly, it has been suggested that NF- $\kappa$ B deficiency or its inhibition *in vivo* should reduce inflammatory responses.

Numerous studies have investigated the molecular mechanisms by which alterations in NF- $\kappa$ B signalling in diverse key cellular processes, including cell proliferation, cell survival, cellular stress response, innate immunity and inflammation are likely to contribute to disease pathology [53]. In this respect, type I diabetes, atherosclerosis, cancer, inflammatory bowel disease (IBD), gastritis, rheumatoid arthritis, systemic lupus erythematosus, asthma, acute respiratory distress syndrome, sepsis and systemic inflammatory response syndrome, surgical major trauma, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and multiple sclerosis are human diseases in which experimental data support a causative role of activation of the NF- $\kappa$ B pathway [52]. The key role of NF- $\kappa$ B in inflammatory responses and immune homeostasis is the basis for the search of compounds targeting any step leading to the nuclear translocation of NF- $\kappa$ B to treat diseases with an inflammatory component.

The beneficial effects of CS for the treatment of OA raise the hypothesis that CS might be effective in other chronic inflammatory processes or diseases because of an autoimmune response. However, in order to accept the hypothesis that CS has a beneficial effect in diseases other than OA, the following question must be answered: can CS inhibition of NF- $\kappa$ B nuclear translocation occur in tissues other than the articulation?

There is preliminary *in vivo* evidence supporting an effect of CS on hepatic NF- $\kappa$ B nuclear translocation and nitric oxide concentrations; administration of CS (20 mg/kg/day) for 20 and 30 days did not affect NF- $\kappa$ B nuclear translocation in healthy rabbits. However, CS prevented the increase of NF- $\kappa$ B nuclear translocation and in nitric oxide hepatic concentrations triggered by a turpentine-induced inflammatory [54]. These results confirm *in vivo* that CS prevents NF- $\kappa$ B activation induced by an inflammatory reaction in tissues other than the articulation. The fact that CS modulates NF- $\kappa$ B in several tissues, such as chondrocytes, synovial membrane and liver, supports the hypothesis that CS could be of benefit in the treatment of other diseases with an inflammatory or autoimmune component.

## Chondroitin sulphate in psoriasis

Deregulation of NF- $\kappa$ B appears to play an important role in skin pathology, such as psoriasis, inflammatory processes like incontinentia pigmenti, Lyme disease, allergic contact dermatitis and autoimmune diseases as well as in skin carcinogenesis [55]. Total expression of the proteins forming NF- $\kappa$ B, the heterodimer p50 and p65, may not be increased in psoriatic lesions [56], but the active phosphorylated form or nuclear expression of NF- $\kappa$ B is detected in 66% of psoriatic lesions and overexpressed in psoriasis

compared with normal skin [57]. In addition, NF- $\kappa$ B function appears deregulated, in the sense that NF- $\kappa$ B DNA binding to the p53  $\kappa$ B site is decreased, whereas NF- $\kappa$ B binding to the pro-inflammatory interleukin-8 (IL-8)  $\kappa$ B site is increased in lesional psoriatic skin compared with non-lesional psoriatic skin [58]. Moreover, the NF- $\kappa$ B-dependent pro-inflammatory cytokines, IL-1 $\beta$  and TNF- $\alpha$ , have a crucial role in the appearance and progression of psoriasis and psoriatic arthritis [59, 60].

The relevancy of NF- $\kappa$ B in psoriasis is supported by a hospital-based, case-control study, including 519 patients with psoriasis *vulgaris* and 541 matched controls who were genotyped for NFKB1 (encodes for p50 protein) polymorphisms. An association between NFKB1 wild-type genotype and an increased risk for psoriasis *vulgaris* was found, for example mutations of the gene NFKB1 reduce NF- $\kappa$ B activity and incidence of psoriasis. The association was more evident in the subgroups of onset age  $\leq$ 40 years, Psoriasis Area and Severity Index (PASI) score  $>$ 20 and male patients [61].

Further supporting the role of NF- $\kappa$ B in psoriasis are the reports showing that effective treatment of psoriasis diminishes NF- $\kappa$ B nuclear translocation. For instance, one study showed that compared with normal epidermis, active phosphorylated NF- $\kappa$ B/RelA in the epidermis from psoriatic plaques was significantly up-regulated, and etanercept, a recombinant human TNF receptor fusion protein, produced a significant down-regulation of phosphorylated NF- $\kappa$ B/RelA, reduction that correlated with decreases in epidermal thickness, restoration of normal markers of keratinocyte differentiation and clinical outcomes [62].

The iminobenzoxathiolone compound 6-hydroxy-1,3-benzoxathiol-2-one is effective for the treatment of psoriasis [63], probably because iminobenzoxathiolones inhibit NF- $\kappa$ B activation and nuclear translocation [64]. Furthermore, the anti-psoriatic effect of avarol-3'-thiosalicylate [65], dimethyl fumarate [66], curcumin [67] and tacrolimus [68] depend upon the down-regulation of NF- $\kappa$ B activity.

In an open non-controlled trial, 11 patients with knee OA and long-standing, moderate-to-severe psoriasis resistant to conventional therapy were treated with 800 mg/day of CS for 2 months. Skin biopsies were obtained before and after treatment. All patients but one improved the condition of the skin, with reduction of swelling, redness, flaking and itching, increase in the hydration and softening of the skin and amelioration of scaling. Histopathologically, CS decreased epidermal thickness, the thickness between the *stratum basale* and the *granulosum*, reduced the number of keratinocytes and diminished the severity of psoriasis activity [69, 70]. These results strongly suggest that CS might be a helpful tool to treat moderate-to-severe psoriasis; however, controlled double-blinded prospective studies have to be conducted to confirm this report.

## Chondroitin sulphate in atherosclerosis

Several reasons prompted researchers to explore whether GAGs are effective in preventing atherosclerosis. Because CS and

heparin are GAGs, it was assumed that CS had antithrombotic properties, and as early as in 1955, Kurita reported that intravenous injections of 5 mg/kg chondroitin sulphate C (CSC – mainly composed of  $\Delta$ di-6S) inhibited the progression of atherosclerosis in rabbits fed with a diet rich in cholesterol [71]. In the early 1960s, Murata confirmed that high doses of sulphated polysaccharides had antithrombotic properties in animal models and affected the progression of atherosclerosis [72, 73]. With ageing and with advanced atherosclerosis, in the arterial wall there is a decrease in chondroitin sulphate A (CSA – mainly composed of  $\Delta$ di-4S) and/or CSC, with a concomitant increase in chondroitin sulphate B (CSB – mainly composed of sulphate O-linked in positions 2 of the glucuronic acid and 4 of the N-acetyl-D-galactosamine), hence it was hypothesized that administration of CSA might prevent the progression of atherosclerosis [74].

A clinical trial compared the evolution of 60 patients with coronary heart disease given initially 10 g daily of CSA for 3 months, followed by doses of 1.5 to 3 g daily for 6 to 30 months with the evolution of 60 patients with coronary heart disease who were not taking CSA. At the end of the trial, three patients of the CSA-treated group presented a coronary event, compared with 21 patients in the control group; however, cardiovascular mortality did not differ [75]. The same patients were followed for 6 years with a dose of CSA reduced by half, for example 0.75 to 1.5 g; after 6 years of treatment with CSA, 6 patients (10%) presented an acute cardiac event, of which four died, compared with 42 patients (70%) of which 14 died in the control group [76]. No adverse effects or abnormal laboratory were found in patients receiving CSA for 6 years. These long-term studies in human beings warrant further double-blind randomized placebo-controlled trials.

There is strong evidence that the incidence and progression of atherosclerosis and subsequent cardiovascular diseases, for example myocardial infarction and stroke, are closely associated to inflammation. Pro-inflammatory cytokines and cellular adhesion molecules involved in the attachment of monocytes to the endothelial wall are critical in early atherogenesis and atherosclerotic lesions are infiltrated with cellular components associated with inflammation; furthermore, in response to acute ischaemia, there is an influx of neutrophils into the walls of the epicardial vessels, and the sites of acute plaque rupture are preferentially associated with inflammatory components [77]. The Cholesterol and Recurrent Events (CARE) trial demonstrated that the presence of inflammation after a myocardial infarction is associated with increased risk of recurrent coronary events [78]. There is growing evidence supporting that pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  play a crucial role in the disruption of macrovascular and microvascular circulation both *in vivo* and *in vitro* [79]. Moreover, markers of inflammation are highly predictive of cardiovascular events [80].

Keeping in mind the role of inflammation on vascular dysfunction, how CS might exert a beneficial effect on the progression of atherosclerosis and cardiovascular pathology? In first place, there is strong evidence that NF- $\kappa$ B is a pivotal transcription factor in the heart and cardiovascular system [81, 82]. The beneficial effect

of acetylsalicylic acid (ASA) on cardiovascular events has been in part explained by a reduction in NF- $\kappa$ B nuclear translocation [83]. RANKL and its soluble decoy receptor OPG modulate vascular calcification, as demonstrated by several reports: (i) mice genetically deficient in OPG show a vascular calcification phenotype, (ii) in calcified arteries, the expression of RANKL is increased and that of OPG is diminished and (iii) there is a clinical association between coronary disease and serum OPG and RANKL levels [84]. The mechanism underlying the role of RANKL/OPG in atherosclerosis appears to be associated with the fact that binding of RANKL to its cognate receptor RANK activates NF- $\kappa$ B nuclear transcription, which stimulates osteoclastic differentiation in preosteoclasts and induces BMP-2 expression in chondrocytes; on the other hand, OPG reduces the effect of RANKL [85].

In summary, there is evidence that in human beings the formation of the arterial atherosclerotic plaque and the progression of atherosclerosis is closely modulated by local and systemic inflammatory reactions, where NF- $\kappa$ B and pro-inflammatory cytokines play a pivotal role. We postulate that CS might limit the progression of atherosclerosis [71–76] by diminishing NF- $\kappa$ B nuclear translocation [14] and by increasing the ratio of OPG/RANKL [29].

A recent study [86] examined the effect of CS on vascular injury and on markers of systemic inflammation in a rabbit model of atherosclerosis aggravated by systemic inflammation provoked by chronic antigen-induced arthritis [87]. Administration of CS prophylactically reduced serum concentrations of C-reactive protein and IL-6. Likewise, CS inhibited the expression of chemokine ligand 2 (CCL2)/monocyte chemoattractant protein-1 (MCP-1) and COX-2, and reduced the nuclear translocation of NF- $\kappa$ B in peripheral blood mononuclear cells. In femoral lesions, CS diminished the expression of CCL2 and COX-2, as well as the ratio of the intima/media thickness. Moreover, CS reduced the percentage of rabbits that developed vascular lesions in the aorta. These results support that CS may prevent/limit the progression of atherosclerosis, probably by diminishing local and systemic inflammatory reactions.

Recently, the possibility was raised that therapies targeting chronic low-grade inflammation may provide novel future strategies for cardiovascular disease prevention [88]. The data presented here support that CS might be a candidate to be used in the prevention of atherosclerotic cardiovascular events. Indeed, randomized, double-blind placebo-controlled trials have to be conducted to demonstrate such hypothesis.

## Chondroitin sulphate in IBD

IBD, ulcerative colitis (UC) and Crohn's disease (CD), are chronic, relapsing gastro-intestinal disorders (GI) disorders of unknown aetiology. IBD is caused by the hyper-activation of effector immune cells through toll-like receptors (TLRs), primarily in macrophages, which produce high levels of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IFN, resulting in colonic tissue damage [89]. NF- $\kappa$ B activation is markedly induced in IBD patients and through its ability to promote the expression of various

pro-inflammatory genes, NF- $\kappa$ B strongly influences the course of mucosal inflammation [90]. Moreover, the NF- $\kappa$ B-dependent cytokines TNF- $\alpha$  and IL-1 $\alpha$  are essential to the inflammatory reaction in UC and CD [91, 92].

Keeping in mind that IBD results from the activation of TLRs and NF- $\kappa$ B, and excessive production of TNF- $\alpha$  and IL-1 $\beta$ , treatment of IBD at each one of these levels has been proposed. To date, there is no effective tool to block directly TLRs [93], but there is preliminary evidence that the activation of the peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ), a natural TLRs suppressor and antagonist, by thiazolidinedione ligands, for example troglitazone, pioglitazone and rosiglitazone, reduces colonic inflammation [94]. The murine dextran sulphate sodium-induced colitis (DSS-IC) is significantly attenuated by pioglitazone and netoglitazone, both prophylactically and therapeutically [95]. A recent multi-centre, randomized, double-blind, placebo-controlled clinical trial compared the efficacy of rosiglitazone with placebo for 12 weeks in 105 patients with mild to moderately active UC; after 12 weeks of therapy, 23 patients (44%) treated with rosiglitazone and 12 patients (23%) treated with placebo achieved clinical response; remission was achieved in nine patients (17%) treated with rosiglitazone and one patient (2%) treated with placebo, and moreover, quality of life was improved in patients receiving rosiglitazone. The authors concluded that rosiglitazone was effective in the treatment of mild to moderately active UC [96].

There is evidence that in intestinal biopsies from patients with CD, the phosphorylation of NF- $\kappa$ B is considerably increased [97], as is the concentration of TNF- $\alpha$  and IL-1 $\beta$  [98]. Oxymatrine (OMT) and matrine (MT) are quinolizidine alkaloids with anti-inflammatory properties that improve the DSS-IC by reducing serum levels of TNF- $\alpha$  and the expression of NF- $\kappa$ B in colonic mucosa [99]. Phytosteryl ferulates are hydroxycinnamic acid derivatives that partially prevent the DSS-IC very probably because they inhibit NF- $\kappa$ B activation [100]. Based on the evidence brought by studies in animals using genetic approaches to inhibit NF- $\kappa$ B activity, it was proposed that blocking NF- $\kappa$ B might offer particular promise to treat IBD [101]. A recent Cochrane review concluded that TNF- $\alpha$  blocking agents, infliximab, CDP571, adalimumab and certolizumab, were effective for the maintenance of remission in patients with CD who have responded to induction therapy [102]. Moreover, current data suggest that infliximab is an effective treatment for patients with moderate-to-severe UC with an inadequate response to conventional glucocorticoid treatment [103].

Is it reasonable to speculate that CS by inhibiting NF- $\kappa$ B phosphorylation and TNF- $\alpha$  and IL-1 $\beta$  release could be beneficial in patients with IBD? There is preliminary evidence suggesting that this may be the case. Using the model of DSS-IC, it was shown that CS improved the symptoms of bloody stools, erosion and increased white blood cells more effectively than 5-aminosalicylic acid [104]. However, more recently, Ota *et al.* showed a mild but non-significant effect of CS on DSS-IC, and in contrast a salmon-derived PG elicited a significant beneficial effect [105]. Interestingly, with the same DSS-IC model, it was shown that another naturally occurring monosaccharide, glucosamine, improved the clinical symptoms and suppressed colonic inflammation and

tissue injury; moreover, glucosamine inhibited the activation of intestinal epithelial cells, as demonstrated by the reduced activation of NF- $\kappa$ B in the intestinal mucosa [106]. The experimental evidence reviewed support the hypothesis that CS might reduce the incidence and severity of relapse of IBD in human beings.

### Chondroitin sulphate in degenerative diseases of the central nervous system (CNS)

There is strong evidence that uncontrolled neuroinflammatory processes, where activated astrocytes and microglia and their cytotoxic agents have a crucial role, contribute to the cascade of events leading to neuronal cell death, as occurs in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease [107].

The neuropathological hallmarks of Alzheimer's disease include deposits of amyloid- $\beta$  peptide (A $\beta$ ), formation of A $\beta$  plaques, accumulation of abnormal tau protein filaments in neurofibrillary tangles, extensive neurodegeneration and signs of chronic inflammation. The A $\beta$  peptides activate NF- $\kappa$ B, resulting in the up-regulation of pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ , NOS-2, and COX-2 [108, 109]. However, there is debate on whether the inflammation contributes to neurotoxic effects or represents a secondary reaction to A $\beta$  deposition. Several, but not all non-steroidal anti-inflammatory drugs (NSAIDs) reduce A $\beta$  burden in transgenic mouse models of Alzheimer's disease, effect that may be mediated by either inhibition of COX or by a direct effect on  $\gamma$ -secretase, thereby reducing A $\beta$  generation independently of COX inhibition [110].

Most epidemiological studies suggest that the risk of suffering Alzheimer's disease is reduced in patients treated with NSAIDs [111]. However, clinical trials on anti-inflammatory drugs including prednisone, hydroxychloroquine and the selective COX-2 inhibitors celecoxib and rofecoxib, showed no effects on cognition. The first large-scale clinical trial on both non-selective and COX-2 selective NSAIDs was also disappointing [112]. A possible explanation is that these drugs might be protective only at the initial stages of the disease, or even before disease initiation, but may not reverse the degenerative process in patients with established pathology.

The PPARs are a family of nuclear hormone receptors that regulate immune and inflammatory responses, with an anti-inflammatory effect. Thiazolidinone derivatives, PPAR $\gamma$  agonists, are potent neuroprotector compounds. Thiazolidinones inhibit the inflammatory activation of cultured brain astrocytes and microglia by diminishing lipopolysaccharide (LPS)-induced IL-6, TNF- $\alpha$ , NOS-2 and COX-2 expression. Neuroprotective effects of thiazolidinones are completely inhibited by PPARs antagonists [113]. Clinical trials showed that rosiglitazone improves cognitive functions in apolipoprotein E4 (ApoE4)-negative patients [114]. Actually, there is evidence that reducing local inflammation with PPAR agonists improves the evolution of diseases such as Alzheimer's and Parkinson [115, 116], confirming that inflammation is a target for treatment.

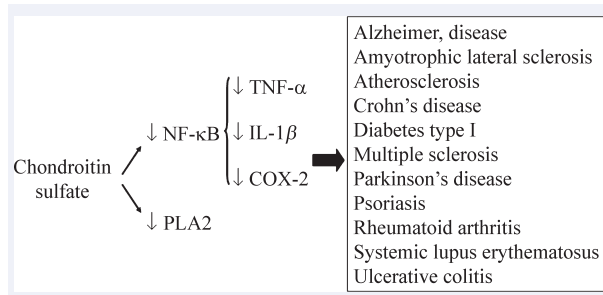
In the CNS, synthesis of matrix CS by macrophages and oligodendrocyte progenitors is strongly up-regulated after a trauma [117]; CS inhibits post-injury axonal regeneration [118]. The CS growth inhibitory effect is considered to be a major obstacle for regeneration, and explain in part the lack of effective CNS recovery [119]. Interestingly, inhibition of CS synthesis immediately after injury impairs functional motor recovery and increases tissue loss, however, allowing CS synthesis during 48 hrs following injury, with subsequent inhibition, improves recovery by directly activating microglia/macrophages *via* the CD44 receptor [120].

The  $\Delta$ di-6S disaccharide modulates neuronal and microglial activities; *in vitro*, in neurons,  $\Delta$ di-6S promotes neurite outgrowth and protects against neuronal toxicity and axonal collapse *via* protein kinase C $\alpha$  (PKC $\alpha$ ) and proline-rich tyrosine kinase 2 (PYK2) intracellular signalling pathways; in microglia,  $\Delta$ di-6S transforms the phenotype of microglia in neuroprotective by means of the activation of Erk1/2 and PYK2. It is noteworthy that *in vivo*, systemically or locally injected  $\Delta$ di-6S protects neurons in mice subjected to glutamate or aggregated  $\beta$ -amyloid intoxication [121, 122]. Recent evidence shows that by regulating multiple genes,  $\Delta$ di-6S confers to microglia behaviour a phagocytic and anti-inflammatory profile [123]. The  $\Delta$ di-6S may be a promising candidate for pharmacological development as a neuroprotective therapy for acute and chronic neurodegenerative disorders [124].

Glycosaminoglycans bind A $\beta$  and can promote its aggregation [125]. The synthetic glycosaminoglycan 3-amino-1-propanesulfonic acid (tramiprosate) was designed to interfere with the binding of glycosaminoglycans and A $\beta$ . This interference should prevent conformational transitions that lead to the assembly of oligomers, protofibrils and fibrils, which ultimately results in plaque deposition. *In vitro*, tramiprosate has demonstrated anti-amyloid activity, including inhibition of A $\beta$ -induced neurotoxicity in neuronal cell cultures. In a phase II clinical trial in patients with mild-to-moderate Alzheimer's disease, tramiprosate decreased the CSF A $\beta$ <sub>42</sub> levels, reflecting a decrease of A $\beta$  in the brain. However, there were no differences in the cognitive and clinical assessments between patients treated with tramiprosate or placebo, probably because 3 months follow-up is too short to record differences [126].

In human neuroblastoma SH-SY5Y cells subjected to oxidative stress, CS reduces the formation of reactive oxygen species (ROS) induced by both H<sub>2</sub>O<sub>2</sub> (extracellular ROS) and Rot/oligo (intracellular ROS), effects associated with an increased AKT/PI3K phosphorylation and heme oxygenase-1 expression [127]. These studies are of particular interest, because it is known that antioxidants elicit a small beneficial effect on the evolution of Alzheimer's and Parkinson diseases [67, 128, 129].

It has been proposed that glaucoma may be considered as a neurodegenerative disease and treated as other neurodegenerative diseases are treated [130]. *In vivo*, the  $\Delta$ di-6S disaccharide protects retinal ganglion cells from death caused by elevated intraocular pressure in part through its control of microglial activity, for example  $\Delta$ di-6S disaccharide activated the microglia through the activation of Erk1/2 and PYK2 but without increasing TNF- $\alpha$  secretion [131].



**Fig. 3** Chondroitin sulphate (CS) by inhibiting the nuclear translocation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B), and consequently, the synthesis of pro-inflammatory cytokines, for example TNF- $\alpha$  and IL-1 $\beta$ , and of pro-inflammatory enzymes, for example cyclooxygenase-2 (COX-2) and phospholipase A2 (PLA2), may be of benefit for multiple autoimmune diseases.

Following the intravenous administration of  $^{131}$ I labelled CS to rats, CS and disaccharides are found in the brain at similar concentrations, for example 0.2 and 0.3  $\mu$ g/g, respectively, compared with blood concentrations of 1.9 and 0.4  $\mu$ g/ml respectively; on the other hand, following oral administration of  $^{131}$ I labelled CS, brain concentrations of CS and disaccharides were 0.2 and 2.3  $\mu$ g/g, respectively, compared with 2.1 and 6.3  $\mu$ g/ml in blood [132]. In agreement with these results, systemic administration of the  $\Delta$ di-6S disaccharide to mice elicits directly or indirectly an effect in the CNS [121, 122, 132]. These studies demonstrate that CS and its disaccharides penetrate the brain when given systemically.

Taken together, because of the anti-inflammatory and antioxidant effects of CS, further animal and human studies are warranted to determine whether glycosaminoglycans could become a new therapeutic strategy for neurodegenerative diseases.

### Other autoimmune diseases that may benefit from chondroitin sulphate

The physiopathology of multiple sclerosis (MS) is complex and still incompletely characterized, but there is growing evidence that several factors contribute to the autoimmune response in MS lesions. MS is an autoimmune demyelinating disease of the CNS primarily mediated by Th1, Th2 and/or Th17 cells, which cross the blood-brain barrier [133, 134]. There is compelling evidence that the increase of Th17 by IL-6 plays a pivotal role in the appearance of autoimmunity, closely associated with the release of IL-17 [134, 135]. IL-17 induces the release of IL-1 $\beta$  and TNF- $\alpha$  from macrophages [136], by activating Erk1/2, p38MAPK and NF- $\kappa$ B [137].

In actively demyelinating plaques, the RelA, c-Rel and p50 subunits of NF- $\kappa$ B are all present in macrophage nuclei in both parenchymal and perivascular areas; RelA is also found in the

nuclei of a subset of hypertrophic astrocytes, suggesting that activation of NF- $\kappa$ B has a role in the evolution of MS [138]. In active MS lesions, NF- $\kappa$ B and JNK are up-regulated in oligodendrocytes located at the edge of active lesions and in microglia/macrophages throughout plaques [139]. There is evidence that NF- $\kappa$ B plays a central role in triggering molecular events in T cells responsible for acute relapse of MS [140].

The most abundant gene transcript present in early active MS lesions is  $\alpha$ B-crystallin (CRYAB), whereas it is absent in normal brain tissue [141]. CRYAB has anti-apoptotic and neuroprotective functions [142], and it is the major target of CD4 $^{+}$  T-cell immunity to the myelin sheath from MS brain [143]. Astrocytes null for CRYAB display an activation of Erk1/2 and p38MAPK and up-regulated expression of NF- $\kappa$ B active subunits p65 and p105/p50, whereas the negative regulator I $\kappa$ B $\alpha$  is down-regulated; in addition, mice Cryab $^{-/-}$  show significantly higher proliferation and secretion of the Th1 cytokines, for example IL-2, IFN- $\gamma$ , TNF- $\alpha$  and IL-12 and Th17 cytokines, for example IL-17 [144].

Taken together, the information available suggest that Th17, IL-17, the phosphorylation of Erk1/2 and p38MAPK and the activation of NF- $\kappa$ B nuclear translocation with further production and release of pro-inflammatory cytokines are important in the appearance and progression of MS. The sequence of events leading to MS allows raising the hypothesis that CS could block or diminish these events. Several arguments support such hypothesis. Firstly, there is evidence that blocking Th2 and mast cell activation improves the experimental autoimmune encephalomyelitis (EAE), an animal model of human MS [133], and CS is capable to down-regulate Th2 response [145, 146]. Secondly, it has been reported that inhibition of PLA2 and COX-2 delays the onset, prevents the development and reduces the severity of EAE and greatly reduces antigen-induced production of Th1 and Th17-type cytokines associated with autoimmune response [147]; it is well documented that CS down-regulates PLA2 [132] and COX-2 [148, 149]. Finally, glucosamine, a natural glucose derivative and an essential component of glycoproteins and PG, suppresses acute EAE, diminishing CNS inflammation and demyelination, effect probably associated with the blockade of Th1 response [150]. This review supports the hypothesis that CS might be an agent beneficial for the treatment of MS, and prompts further studies.

### Conclusions

Despite the limitations of the *in vitro* and *in vivo* animal models because of differences in CS dosages, routes of administration and duration of exposure, this review supports that by inhibiting nuclear translocation of NF- $\kappa$ B and subsequent production of pro-inflammatory cytokines, and COX-2 and PLA2 expression and activity (Fig. 3), CS might potentially be of interest for the treatment of many inflammatory and autoimmune diseases, besides OA [151, 152]. Being CS a SYSADOA, we predict that an effect of



CS could only be detected on long-term treatments, for example months. Because of the nature, the evolution and the difficulty to treat the inflammatory or autoimmune diseases listed in Figure 3, animal and human studies are warranted to test whether CS provides any beneficial effect.

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