EUR Research Information Portal

Glucosamine in Osteoarthritis, Effect on articular joint tissues

Publication status and date:
Published: 17/02/2010

Document Version
Publisher's PDF, also known as Version of record

Citation for the published version (APA):
Uitterlinden, EJ. (2010). *Glucosamine in Osteoarthritis, Effect on articular joint tissues*. [Doctoral Thesis, Erasmus University Rotterdam]. Erasmus Universiteit Rotterdam (EUR).

Link to publication on the EUR Research Information Portal

Terms and Conditions of Use
Except as permitted by the applicable copyright law, you may not reproduce or make this material available to any third party without the prior written permission from the copyright holder(s). Copyright law allows the following uses of this material without prior permission:

- you may download, save and print a copy of this material for your personal use only;
- you may share the EUR portal link to this material.

In case the material is published with an open access license (e.g. a Creative Commons (CC) license), other uses may be allowed. Please check the terms and conditions of the specific license.

Take-down policy
If you believe that this material infringes your copyright and/or any other intellectual property rights, you may request its removal by contacting us at the following email address: openaccess.library@eur.nl. Please provide us with all the relevant information, including the reasons why you believe any of your rights have been infringed. In case of a legitimate complaint, we will make the material inaccessible and/or remove it from the website.

Erasmus University Rotterdam
Making Minds Matter

Glucosamine in Osteoarthritis

Effects on Articular Joint Tissues

Elian Uitterlinden

Cover design: Optima Grafische Communicatie, Rotterdam
Layout: Optima Grafische Communicatie, Rotterdam
Printing: Optima Grafische Communicatie, Rotterdam

ISBN: 978-90-8559-609-7

© E.J. Uitterlinden, The Netherlands, 2009. All rights reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, without prior written permission of the author or, when appropriate, of the scientific journal in which parts of this thesis have been published.

Glucosamine in Osteoarthritis

Effects on Articular Joint Tissues

Glucosamine bij artrose

effecten op gewrichtsweefsels

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 17 februari 2010 om 13.30 uur

door

Elias Jan Uitterlinden

geboren te Dordrecht

ERASMUS UNIVERSITEIT ROTTERDAM

PROMOTIECOMMISSIE

Promotoren:
Prof.dr. J.A.N. Verhaar
Prof.dr.ir. H. Weinans

Overige leden:
Prof.dr. A.G. Vulto
Prof.dr. P.J.E. Bindels
Prof.dr. J.W.J. Bijlsma

Copromotor:
Dr. G.J.V.M. van Osch

CONTENTS

	List of Abbreviations	6
Chapter 1	General Introduction	7
Chapter 2	Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants	21
Chapter 3	Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate	37
Chapter 4	Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants	55
Chapter 5	Effect of glucosamine sulphate on joint space narrowing, pain and function in patients with hip osteoarthritis; subgroup analyses of a randomized controlled trial	67
Chapter 6	Does oral glucosamine detectably alter gene expression in cartilage and bone? An explorative pilot study in patients with hip osteoarthritis	79
Chapter 7	Summary & General Discussion	97
Chapter 8	Nederlandse samenvatting	113
Appendices	Dankwoord	123
	Curriculum Vitae	127
	PhD Portfolio	129
	List of Publications	131
	The effect of glucosamine sulphate on osteoarthritis: design of a long-term randomised clinical trial	133

LIST OF ABBREVIATIONS

ADAMTS	A Desintegrin and a Metalloproteinase with Thrombospondin Motif
ATP	Adenosine Triphosphate
CS	Chondroitin Sulphate
DEXA	Dual Energy X-ray Absorptiometry
DMEM	Dulbecco's Modified Eagle Medium
DMOAD	Disease-Modifying Osteoarthritis Drug
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
FCS	Fetal Calf Serum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GlcN	Glucosamine
GlcN-Ac	N-acetyl-glucosamine
GlcN-HCl	Glucosamine-hydrochloride
GlcN-S	Glucosamine-sulphate
Gluc	Glucose
HA	Hyaluronic Acid
HAS	Hyaluronic Acid Synthase
IL	Interleukin
JSN	Joint Space Narrowing
JSW	Joint Space Width
KL	Kellgren & Lawrence
MMP	Matrix Metalloproteinase
NSAID	Non-Steroidal Anti-Inflammatory Drug
OA	Osteoarthritis
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SD	Standard Deviation
TIMP	Tissue Inhibitors of Matrix Metalloproteinase
VAS	Visual Analogue Scale
WOMAC	Western Ontario McMaster Universities

Chapter 1

General Introduction

OSTEOARTHRITIS

Osteoarthritis (OA) is a disease of the articular joints that can lead to severe invalidation. With the aging of the population, increasing numbers of persons will become affected by this condition [1]. The burden of musculoskeletal disease is also rising, with OA being the major cause of this burden [2]. Although the exact etiology of OA is not known, risk factors such as being overweight, physical workload and high-intensity sporting activities have been identified [1]. Patients suffering from OA often consult healthcare professionals at different disease stages, but the common complaints are pain, or loss of function of the affected joint. OA can be present in every joint, with large differences in prevalence rates.

With regard to the major joints, symptomatic OA is mostly found in the knee and hip [3]. Although the hip is a ball-and-socket joint and the knee is a hinge joint (thus biomechanically they are different), they are both synovial joints. A synovial or diarthrodial joint enables two bones to articulate relative to each other. In general, a synovial joint is composed of two bone ends that are covered by articular cartilage and encapsulated by a fibrous capsule. The articular side of this capsule is covered by the synovial membrane that produces synovial fluid. This synovial fluid provides nutrients to the cartilage and serves as a lubricant of the joint.

In lay terms, OA is mistakenly translated as 'cartilage wear'. The term 'wear' implies that cartilage tissue will deteriorate during normal daily use, but that certainly is not the case. OA is a far more complex disease than simply the wearing down of cartilage tissue. In OA all joint components (i.e., cartilage, synovium and bone) will at some stage be involved in the disease process (Fig. 1). Apart from the involvement of the components directly present within the

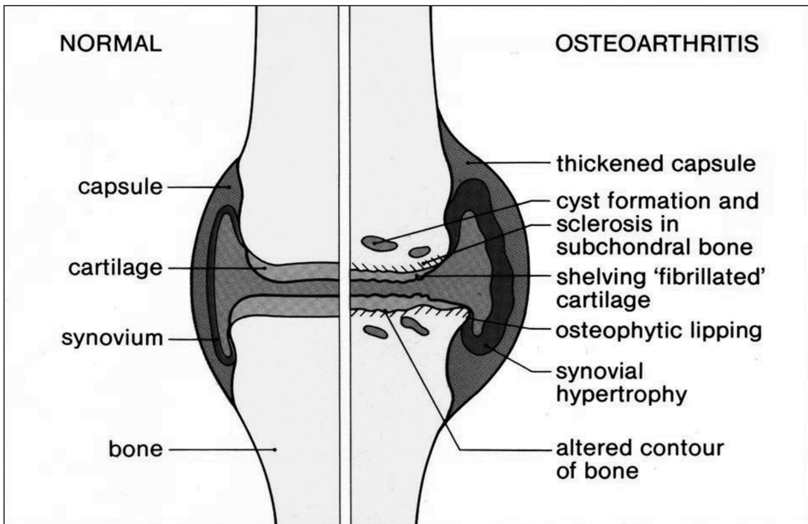

Figure 1. Sketch of a typical joint. The left side shows the tissues that form a normal synovial joint. The right side shows the changes that can occur in these tissues in the presence of osteoarthritis. Figure reproduced with permission from D.T. Felson.

joint, other structures such as muscles and ligaments surrounding the joint can also become affected [4].

CARTILAGE

Articular or hyaline cartilage is a highly specialized tissue that covers both ends of two articulating bones. Cartilage, together with synovial fluid, provides almost frictionless movement of the two articulating bones within a joint [5]. Cartilage contains no vascular, neural or lymphatic tissue and is metabolically dependent on the surrounding synovial fluid and the underlying subchondral bone [5,6]. Therefore, cartilage functions in an environment of very low oxygen tension and relies on anaerobic metabolism. Cartilage is built up of chondrocytes, their surrounding extracellular matrix (ECM), and water. The ECM is produced by the chondrocytes themselves. Production of ECM is, for example, needed during growth of the long bones by the mechanism of endochondral bone formation, and to maintain cartilage tissue integrity [7,8]. The cartilage ECM consists of proteoglycans that are surrounded by a collagen framework. Two thirds of the dry weight of adult cartilage consists of collagens. Collagen type II is by far the most abundant collagen type, along with collagen types III, VI, IX, X, XI, XII and XIV [9]. Proteoglycans are macromolecules that consist of a core protein with covalently bound carbohydrate side chains. These carbohydrate side chains are known as glycosaminoglycans (GAGs). In the nucleus of the chondrocyte, messenger RNA is transcribed from the DNA of the proteoglycan genes. In the rough endoplasmic reticulum the messenger RNA is translated to form the protein core. This protein is synthesized at the ribosome and then transported to the Golgi complex. Here the GAG chains are covalently joined to the core protein, one sugar at a time in repeating disaccharide units. The repeating disaccharide units are characteristic for each individual GAG. Glucosamine (GlcN) is always present as a building block in these disaccharide units, either directly as GlcN or after a conversion to galactosamine. During the elongation of the carbohydrate chains the hexosamine residues are also being sulfated. Once glycosylation and sulphation is completed, the macromolecules are transported to the cell membrane via secretory vesicles and released into the ECM. Cartilage contains various proteoglycans such as aggrecan, decorin, biglycan, fibromodulin and lumican. The large content of the proteoglycan aggrecan is characteristic of hyaline cartilage [10]. Together with hyaluronic acid (HA) and link protein, aggrecan forms large proteoglycan aggregates in the ECM. Due to the large number of GAGs within these proteoglycan aggregates, a fixed negative charge exists which makes this whole complex strongly hydrophilic. Therefore, water is the major component of the cartilage wet weight, namely 65-75% in adult cartilage [11].

In OA changes take place within the cartilage. Macroscopically, the cartilage structure changes from a smooth surface via softening and swelling, fragmentation and fissuring, to total erosion of cartilage down to the subchondral bone [12]. Microscopically, this can be observed

as destruction of cartilage structure and thus deterioration of the ECM, with the collagen and the proteoglycans becoming degraded. In OA the normal equilibrium between ECM synthesis and degradation becomes distorted in favor of the latter one, leading to a net decrease in the amount of ECM and, in the end, complete disappearance of the articular cartilage [13]. This ECM degradation is influenced by increased activity of proteolytic enzymes, such as the matrix metalloproteinases (MMPs) and the ADAMTSs (a desintegrin and a metalloproteinase with thrombospondin motif) [13,14]. The activity of the MMPs is influenced by the tissue inhibitors of MMPs (i.e., TIMPs) [15,16]. The number of newly identified MMPs is still growing, but the most important ones regarding the OA disease process are MMP3 and 13 [13,17,18]. Within the ADAMTSs, aggrecanase 1 and 2 (ADAMTS4 and 5) seem to be the most important ones [17,19].

SYNOVIUM

The joint wall, or capsule, can be subdivided in two distinct layers. The outer layer which consists of thick dense connective tissue and the inner layer which is called the synovial membrane or synovium. The cell type found in this synovium is the synoviocyte. There are mainly two types of synoviocytes: type A cells (macrophagic cells) and type B cells (fibroblast-like cells). The latter are believed to be the predominant and proper synovial membrane cells [20]. Synoviocytes are responsible for the production of synovial fluid. The synovial fluid serves as a lubricant for the joint, but also plays an important role in joint homeostasis. Synovial fluid mainly contains water and HA [21]. It is thought that HA plays a major role in joint lubrication and in maintaining joint homeostasis [22]. HA is a non-sulphated GAG, built up of alternating units of N-acetylglucosamine and glucuronic acid. It is produced inside the cell at the plasma membrane, and is then secreted directly into the ECM [23].

Although OA is often classed as a non-inflammatory disease, a variable degree of synovitis can be observed in OA [24]. This synovitis is characterized by papillary hyperplasia of the synovial membrane, proliferation of blood vessels and infiltration with mononuclear cells (predominantly T-cells) [25]. It is unclear whether these morphological changes in the synovium are a primary change in the pathogenesis of OA, or are secondary to joint inflammation and cartilage degradation resulting in exposed subchondral bone [26]. The synovitis may lead to an increased production of synovial fluid. This might be noticed by patients and physicians as swelling of the joint and (sometimes) an increased temperature of the joint. If the amount of synovial fluid becomes large enough, mechanical obstruction of joint movement can even develop. It has been hypothesized that the synovium might play an important role in the pathogenesis of OA by the induction of various pro- and anti-inflammatory agents. These inflammatory agents can lead to cartilage degeneration through MMP activation and up-regulation of prostaglandin and nitric oxide. The degradation products of cartilage are able to maintain or worsen the inflammatory process of synovium [24].

BONE

At any time during the OA disease process the bone underlying the cartilage will also become affected. In the clinical setting, bony changes can be directly visualized on an X-ray of the involved joint. On the X-rays of OA joints bony changes (e.g., subchondral sclerosis, bone cyst formation, osteophyte formation and gross deformity of the joint) can be found. In contrast, changes in cartilage in OA can only be detected through a diminished joint space since cartilage itself is radiolucent [27]. Radin and co-workers described that bone had a decreased capacity to absorb energy [28] and an increased stiffness in OA [29]. They were the first ones who proposed a link between subchondral bone mechanics and OA progression [30,31]. They hypothesized that an initiating factor in OA was the stiffening of the subchondral plate. More recently, Imhof et al. [6] proposed that in degenerative joint disease microcracks, microedema and microbleeding occur within the subchondral plate due to repetitive overloading. When the subsequent healing process is insufficient and vascularity becomes compromised, degenerative changes can develop. It is thus believed that cartilage and subchondral bone should be considered an interdependent functional unit [32].

TREATMENT

As with many diseases in the human body, treatment modalities can be subdivided into two main groups: operative treatment and conservative (non-operative) treatment.

An example of operative treatment is a corrective osteotomy (e.g., varus, valgus, abduction and adduction osteotomy) used to change the loading pattern to relieve symptoms and postpone more definitive operative treatment, like arthrodesis or joint replacement surgery. At the hip, joint replacement surgery can be performed using articular surface replacement prosthesis or a total hip prosthesis, uncemented, fully cemented or using hybrid fixation (one part cemented, the other uncemented). In knee OA choices can be made between unicompartmental, patellofemoral or total knee prosthesis. Total hip and knee arthroplasty are commonly performed and relatively successful procedures in orthopaedic surgery, with 22,500 total hip and 10,329 total knee replacements being performed in the Netherlands in 2005 [33,34]. Another (not yet commonly performed) type of operative treatment is joint distraction. In severe ankle OA long-term clinical benefit was shown using Ilizarov joint distraction [35].

Conservative treatment mainly focuses on symptom relief. OA-related pain can be treated using pain medication, such as paracetamol or non-steroidal anti-inflammatory drugs. In severe cases, pain and joint inflammation can be treated with an intra-articular injection of a corticosteroid. Promising beneficial effects on pain and function were also found with intra-articular injection of HA in patients with knee OA [36]. Apart form pharmaceutical interventions, exercises are effective in improving joint stiffness, restoring muscle function, and regaining and

retaining a certain level of activity. The use of braces or orthoses can sometimes relieve OA-related symptoms [37]. In addition to the above-mentioned conservative treatments, physicians should advise their patients to adapt their activity level to their complaints and, if applicable, to reduce bodyweight.

All these treatment modalities should be regarded as treatment of the symptoms of OA, none of them actually heals OA. The search for a compound that can directly influence the course of OA, a so-called 'disease-modifying osteoarthritis drug', has received increasing attention over the last decades. Glucosamine (GlcN), a food additive, is one of these alleged disease-modifying osteoarthritis drugs, and is the main topic of investigation in this thesis.

GLUCOSAMINE

GlcN, the food additive with supposed disease-modifying activity, is also referred to as a nutraceutical (i.e., nutrient with pharmaceutical properties). Since it is a food additive, manufacturers do not have to comply with the same strict regulations of safety, quality assurance and proof of effectiveness that are required in the pharmaceutical industry. Therefore, claims made by the manufacturers about the effectiveness of GlcN on the disease progression of OA should be critically reviewed. This task is best performed by independent research institutions that are not involved with the manufacturer or the manufacturing process.

Although the first report on the use of GlcN with cartilage was published as early as 1956 [38], attention was renewed in 2001 and 2002 with two publications on the possible disease-modifying properties of GlcN in OA. Both these studies provided indications that GlcN decreased radiographic OA disease progression when compared to placebo over a period of several years and thus structure-modification of GlcN was assumed [39,40]. Other groups showed that GlcN also had an effect on symptoms above placebo level in OA [41-43]. On the other hand, some authors could not confirm these findings [44-46]. Moreover, in reviews and meta-analyses, GlcN proved to be safe in use with little or no side-effects [47,48].

In the human body GlcN is endogenously formed from glucose. After phosphorylation and conversion to fructose-6-phosphate, glucosamine-6-phosphate is created using the glutamine:fructose-6-phosphate amidotransferase, with glutamine as the amino donor [49]. Exogenous GlcN gets phosphorylated to glucosamine-6-phosphate by the same enzyme responsible for the phosphorylation of glucose, glucokinase [50]. The phosphorylated form of GlcN can then be used further downstream to produce proteoglycans (Fig. 2).

Commercially, GlcN is available as a supplement mainly as glucosamine-sulphate, glucosamine-hydrochloride and N-acetyl-glucosamine. When GlcN is administered orally, gastrointestinal absorption is close to 90%; however, after the first pass effect in the liver bioavailability will be 44% [51]. GlcN reaches the synovial fluid via the systemic circulation. Although some have measured very low synovial GlcN concentrations, studies with radio-labelled GlcN showed a

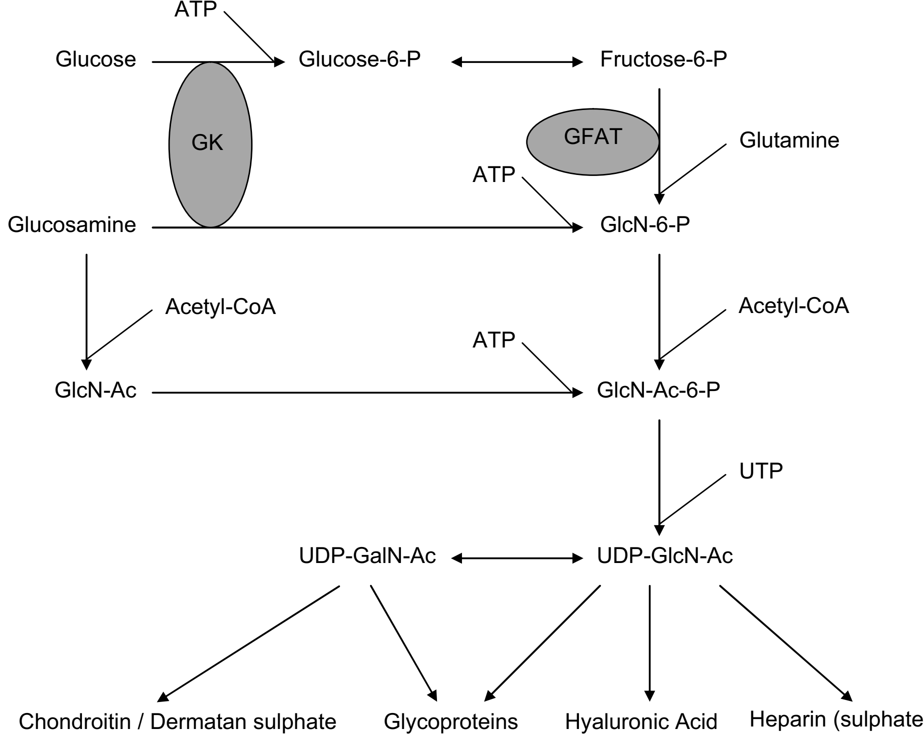

Figure 2. Metabolic pathways of glucosamine. Major enzymes are shown in the shaded ovals. Abbreviations: ATP, adenosine triphosphate; Glucose-6-P, glucose-6-phosphate; Fructose-6-P, fructose-6-phosphate; GK, glucokinase; GFAT, glutamine:fructose-6-phosphate amidotransferase; GlcN-6-P, glucosamine-6-phosphate; Acetyl-CoA, acetyl-coenzymeA; GlcN-Ac, N-acetyl-glucosamine; GlcN-Ac-6-P, N-acetyl-glucosamine-6-phosphate; UTP, uridine triphosphate; UDP-GalN-Ac, uridine diphosphate-N-acetyl-galactosamine; UDP-GlcN-Ac, uridine diphosphate-N-acetyl-glucosamine.

special capacity of articular cartilage to accumulate and retain GlcN [51-54]. Thus, the concentration of GlcN directly surrounding the chondrocytes is believed to be higher than that measured in the synovial fluid.

As stated earlier, GlcN is thought to slow down joint space narrowing and reduce the symptoms found in OA. However, the precise working mechanism of GlcN remains unclear. Some believe that GlcN is effective because it is a building block of GAG, which is important for the maintenance of the ECM of cartilage. Some studies support this hypothesis [55-59]. Other groups, however, could not confirm this effect or even found negative effects on GAG production after GlcN addition [60-67]. Yet others have hypothesized that GlcN has anti-inflammatory properties through interference with enzymatic matrix degradation [56,61,67-72].

AIM AND OUTLINE OF THIS THESIS

Since all current treatment modalities, whether conservative or operative, can only treat the symptoms of OA and not the disease itself, the possibility that GlcN can act as a disease- modifying osteoarthritis drug, should be thoroughly investigated.

The general aim of this thesis is to gain more insight into the working mechanism of GlcN in OA, to be able to provide relevant advice on the use of this compound in patients with OA. The first part of the thesis consists of *in vitro* experiments with different OA models using the tissue types that can be involved in OA. In the second part of the thesis GlcN is administered orally to patients using placebo-controlled methodology, to establish its effects on OA symptoms, and to examine the effects on joint tissue *in vivo* after a period of GlcN ingestion.

In **chapter 2**, the effect of different GlcN derivatives in a human osteoarthritic explant model, using cartilage obtained at total knee replacement surgery, on the expression of genes involved in anabolic and catabolic activities of chondrocytes is investigated. In **chapter 3**, the working mechanism of GlcN is studied by determining the effect of different GlcN derivatives on bovine chondrocytes in a culture model using alginate beads under anabolic and catabolic culture conditions. Here we used interleukin-1β to mimic OA. In **chapter 4**, the effect of GlcN on HA production in a human synovium explant model using synovium obtained at total knee replacement surgery is determined. In **chapter 5**, a subgroup analysis of a larger cohort of 222 primary care patients who received glucosamine-sulphate or placebo over a two-year period is presented, focusing on the effects of GlcN on OA symptoms and progression. The design of this randomized clinical trial can be found in the **Appendix** of this thesis. In **chapter 6**, the effects of pre-operative GlcN use on gene expression of genes involved in anabolic and catabolic activities in human osteoarthritic hip cartilage and bone are described. Finally, in **chapter 7** the results and limitations of these studies are summarized and discussed and implications for future research are presented.

REFERENCES

1. Bierma-Zeinstra SM, Koes BW. Risk factors and prognostic factors of hip and knee osteoarthritis. Nat Clin Pract Rheumatol 2007;3:78-85.
2. Brooks PM. The burden of musculoskeletal disease--a global perspective. Clin Rheumatol 2006;25: 778-81.
3. Petersson IF, Jacobsson LT. Osteoarthritis of the peripheral joints. Best Pract Res Clin Rheumatol 2002; 16:741-60.
4. Felson DT. An update on the pathogenesis and epidemiology of osteoarthritis. Radiol Clin North Am 2004;42:1-9, v.
5. Huber M, Trattnig S, Lintner F. Anatomy, biochemistry, and physiology of articular cartilage. Invest Radiol 2000;35:573-80.
6. Imhof H, Sulzbacher I, Grampp S, Czerny C, Youssefzadeh S, Kainberger F. Subchondral bone and cartilage disease: a rediscovered functional unit. Invest Radiol 2000;35:581-8.
7. Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, *et al.* Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. Development 2004;131:5883-95.
8. Mobasheri A, Vannucci SJ, Bondy CA, Carter SD, Innes JF, Arteaga MF, *et al.* Glucose transport and metabolism in chondrocytes: a key to understanding chondrogenesis, skeletal development and cartilage degradation in osteoarthritis. Histol Histopathol 2002;17:1239-67.
9. Eyre D. Collagen of articular cartilage. Arthritis Res 2002;4:30-5.
10. Roughley PJ. The structure and function of cartilage proteoglycans. Eur Cell Mater 2006;12:92-101.
11. Thompson RC, Jr., Robinson HJ, Jr. Articular cartilage matrix metabolism. J Bone Joint Surg Am 1981; 63:327-31.
12. Outerbridge RE. The etiology of chondromalacia patellae. J Bone Joint Surg Br 1961;43-B:752-7.
13. Nagase H, Kashiwagi M. Aggrecanases and cartilage matrix degradation. Arthritis Res Ther 2003;5: 94-103.
14. Mort JS, Billington CJ. Articular cartilage and changes in arthritis: matrix degradation. Arthritis Res 2001;3:337-41.
15. Somerville RP, Oblander SA, Apte SS. Matrix metalloproteinases: old dogs with new tricks. Genome Biol 2003;4:216.
16. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. J Biol Chem 1999;274:21491-4.
17. Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E, Aigner T. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. Arthritis Rheum 2002;46:2648-57.
18. Bramono DS, Richmond JC, Weitzel PP, Kaplan DL, Altman GH. Matrix Metalloproteinases and Their Clinical Applications in Orthopaedics. Clin Orthop 2004;1:272-85.
19. Malfait AM, Liu RQ, Ijiri K, Komiya S, Tortorella MD. Inhibition of ADAM-TS4 and ADAM-TS5 prevents aggrecan degradation in osteoarthritic cartilage. J Biol Chem 2002;277:22201-8.
20. Iwanaga T, Shikichi M, Kitamura H, Yanase H, Nozawa-Inoue K. Morphology and functional roles of synoviocytes in the joint. Arch Histol Cytol 2000;63:17-31.
21. Curtiss PH, Jr. Changes Produced in the Synovial Membrane and Synovial Fluid by Disease. J Bone Joint Surg Am 1964;46:873-88.
22. Laurent TC, Laurent UB, Fraser JR. The structure and function of hyaluronan: An overview. Immunol Cell Biol 1996;74:A1-7.
23. Prehm P. Hyaluronate is synthesized at plasma membranes. Biochem J 1984;220:597-600.
24. Sutton S, Clutterbuck A, Harris P, Gent T, Freeman S, Foster N, *et al.* The contribution of the synovium, synovial derived inflammatory cytokines and neuropeptides to the pathogenesis of osteoarthritis. Vet J 2009;179:10-24.

25. Fernandez-Madrid F, Karvonen RL, Teitge RA, Miller PR, An T, Negendank WG. Synovial thickening detected by MR imaging in osteoarthritis of the knee confirmed by biopsy as synovitis. Magn Reson Imaging 1995;13:177-83.
26. Loeuille D, Chary-Valckenaere I, Champigneulle J, Rat AC, Toussaint F, Pinzano-Watrin A, *et al.* Macroscopic and microscopic features of synovial membrane inflammation in the osteoarthritic knee: correlating magnetic resonance imaging findings with disease severity. Arthritis Rheum 2005;52: 3492-501.
27. Zacher J, Carl HD, Swoboda B, Backhaus M. [Imaging of osteoarthritis of the peripheral joints] Bildgebung bei der Arthrose peripherer Gelenke. Z Rheumatol 2007;66:257-8, 60-4, 66.
28. Radin EL, Paul IL, Lowy M. A comparison of the dynamic force transmitting properties of subchondral bone and articular cartilage. J Bone Joint Surg Am 1970;52:444-56.
29. Pugh JW, Radin EL, Rose RM. Quantitative studies of human subchondral cancellous bone. Its relationship to the state of its overlying cartilage. J Bone Joint Surg Am 1974;56:313-21.
30. Radin EL, Rose RM. Role of subchondral bone in the initiation and progression of cartilage damage. Clin Orthop Relat Res 1986:34-40.
31. Radin EL, Paul IL, Rose RM. Role of mechanical factors in pathogenesis of primary osteoarthritis. Lancet 1972;1:519-22.
32. Lajeunesse D, Reboul P. Subchondral bone in osteoarthritis: a biologic link with articular cartilage leading to abnormal remodeling. Curr Opin Rheumatol 2003;15:628-33.
33. Prismant. Yeardisk for the hospitals. Utrecht 2005.
34. Statistics Netherlands. Statistical yearbook of the Netherlands. Heerlen 2005.
35. Ploegmakers JJ, van Roermund PM, van Melkebeek J, Lammens J, Bijlsma JW, Lafeber FP, *et al.* Prolonged clinical benefit from joint distraction in the treatment of ankle osteoarthritis. Osteoarthritis Cartilage 2005;13:582-8.
36. Bellamy N, Campbell J, Robinson V, Gee T, Bourne R, Wells G. Viscosupplementation for the treatment of osteoarthritis of the knee. Cochrane Database Syst Rev 2006:CD005321.
37. Brouwer RW, Jakma TS, Verhagen AP, Verhaar JA, Bierma-Zeinstra SM. Braces and orthoses for treating osteoarthritis of the knee. Cochrane Database Syst Rev 2005:CD004020.
38. Rodén L. Effect of hexosamines on the synthesis of chondroitin sulphuric acid in vitro. Arkh Kemi 1956;10:345-52.
39. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, *et al.* Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-6.
40. Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002;162:2113-23.
41. Herrero-Beaumont G, Ivorra JA, Del Carmen Trabado M, Blanco FJ, Benito P, Martin-Mola E, *et al.* Glucosamine sulfate in the treatment of knee osteoarthritis symptoms: a randomized, double-blind, placebo-controlled study using acetaminophen as a side comparator. Arthritis Rheum 2007;56: 555-67.
42. Braham R, Dawson B, Goodman C. The effect of glucosamine supplementation on people experiencing regular knee pain. Br J Sports Med 2003;37:45-9; discussion 9.
43. Houpt JB, McMillan R, Wein C, Paget-Dellio SD. Effect of glucosamine hydrochloride in the treatment of pain of osteoarthritis of the knee. J Rheumatol 1999;26:2423-30.
44. Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, *et al.* Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. N Engl J Med 2006;354:795-808.
45. McAlindon T, Formica M, LaValley M, Lehmer M, Kabbara K. Effectiveness of glucosamine for symptoms of knee osteoarthritis: results from an internet-based randomized double-blind controlled trial. Am J Med 2004;117:643-9.

46. Cibere J, Kopec JA, Thorne A, Singer J, Canvin J, Robinson DB, *et al.* Randomized, double-blind, placebo-controlled glucosamine discontinuation trial in knee osteoarthritis. Arthritis Rheum 2004; 51:738-45.
47. Anderson JW, Nicolosi RJ, Borzelleca JF. Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. Food Chem Toxicol 2005;43:187-201.
48. Towheed T, Maxwell L, Anastassiades T, Shea B, Houpt J, Robinson V, *et al.* Glucosamine therapy for treating osteoarthritis. Cochrane Database Syst Rev 2005:CD002946.
49. Mroz PJ, Silbert JE. Effects of [3H]glucosamine concentration on [3H]chondroitin sulphate formation by cultured chondrocytes. Biochem J 2003;376:511-5.
50. Van Schaftigen E. Glucosamine-sensitive and -insensitive detritiation of [2-3H]glucose in isolated rat hepatocytes: a study of the contributions of glucokinase and glucose-6-phosphatase. Biochem J 1995;308 (Pt 1):23-9.
51. Setnikar I, Rovati LC. Absorption, distribution, metabolism and excretion of glucosamine sulfate. A review. Arzneimittelforschung 2001;51:699-725.
52. Setnikar I, Giacchetti C, Zanolo G. Pharmacokinetics of glucosamine in the dog and in man. Arzneimittelforschung 1986;36:729-35.
53. Setnikar I, Giachetti C, Zanolo G. Absorption, distribution and excretion of radioactivity after a single intravenous or oral administration of [14C] glucosamine to the rat. Pharmatherapeutica 1984;3:538-50.
54. Setnikar I, Palumbo R, Canali S, Zanolo G. Pharmacokinetics of glucosamine in man. Arzneimittelforschung 1993;43:1109-13.
55. Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-34.
56. Dodge GR, Jimenez SA. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis Cartilage 2003;11:424-32.

57. Bassleer C, Henrotin Y, Franchimont P. In-vitro evaluation of drugs proposed as chondroprotective agents. Int J Tissue React 1992;14:231-41.
58. Lippiello L. Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress. Osteoarthritis Cartilage 2003;11:335-42.
59. Vidal y Plana RR, Bizzarri D, Rovati AL. Articular cartilage pharmacology: I. In vitro studies on glucosamine and non steroidal antiinflammatory drugs. Pharmacol Res Commun 1978;10:557-69.
60. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:444-51.
61. Orth MW, Peters TL, Hawkins JN. Inhibition of articular cartilage degradation by glucosamine-HCl and chondroitin sulphate. Equine Vet J Suppl 2002:224-9.
62. de Mattei M, Pellati A, Pasello M, de Terlizzi F, Massari L, Gemmati D, *et al.* High doses of glucosamine-HCl have detrimental effects on bovine articular cartilage explants cultured in vitro. Osteoarthritis Cartilage 2002;10:816-25.
63. Anderson CC, Cook JL, Kreeger JM, Tomlinson JL, Wagner-Mann CC. In vitro effects of glucosamine and acetylsalicylate on canine chondrocytes in three-dimensional culture. Am J Vet Res 1999;60: 1546-51.
64. Ilic MZ, Martinac B, Handley CJ. Effects of long-term exposure to glucosamine and mannosamine on aggrecan degradation in articular cartilage. Osteoarthritis Cartilage 2003;11:613-22.
65. Gouze JN, Bianchi A, Becuwe P, Dauca M, Netter P, Magdalou J, *et al.* Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-kappa B pathway. FEBS Lett 2002;510:166-70.

66. Mroz PJ, Silbert JE. Use of (3)H-glucosamine and (35)S-sulfate with cultured human chondrocytes to determine the effect of glucosamine concentration on formation of chondroitin sulfate. Arthritis Rheum 2004;50:3574-9.
67. Fenton JI, Chlebek-Brown KA, Caron JP, Orth MW. Effect of glucosamine on interleukin-1-conditioned articular cartilage. Equine Vet J Suppl 2002:219-23.
68. Piperno M, Reboul P, Hellio Le Graverand MP, Peschard MJ, Annefeld M, Richard M, *et al.* Glucosamine sulfate modulates dysregulated activities of human osteoarthritic chondrocytes in vitro. Osteoarthritis Cartilage 2000;8:207-12.
69. Sandy JD, Gamett D, Thompson V, Verscharen C. Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. Biochem J 1998;335 (Pt 1):59-66.
70. Gouze JN, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, *et al.* Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis: influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. Arthritis Rheum 2001;44:351-60.
71. Byron CR, Orth MW, Venta PJ, Lloyd JW, Caron JP. Influence of glucosamine on matrix metalloproteinase expression and activity in lipopolysaccharide-stimulated equine chondrocytes. Am J Vet Res 2003;64:666-71.
72. Mello DM, Nielsen BD, Peters TL, Caron JP, Orth MW. Comparison of inhibitory effects of glucosamine and mannosamine on bovine articular cartilage degradation in vitro. Am J Vet Res 2004;65:1440-5.

Chapter 2

Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants

E.J. Uitterlinden, H. Jahr, J.L.M. Koevoet, Y.M. Jenniskens, S.M.A. Bierma-Zeinstra, J. DeGroot, J.A.N. Verhaar, H. Weinans, G.J.V.M. van Osch

Osteoarthritis & Cartilage 2006 Mar; 14(3):250-7

ABSTRACT

Objective

To investigate the effect of glucosamine (GlcN) in a human osteoarthritic explant model on expression of genes involved in anabolic and catabolic activities of chondrocytes.

Methods

Human osteoarthritic explants, obtained during knee arthroplasty surgery, were pre-cultured (3 days) and treated with glucosamine-hydrochloride (GlcN-HCl) or glucosamine-sulphate (GlcN-S) at 0.5 mM and 5 mM (4 days). RNA was isolated from the explants and real time RT-PCR was performed. Additionally, total matrix metalloproteinase (MMP) activity was measured in culture medium.

Results

Addition of 5 mM GlcN led to significant down-regulation of aggrecan (2.65-7.73-fold) and collagen type II (7.75-22.17-fold) gene expression, indicating inhibited anabolic activity. Considering catabolic activities, 5 mM GlcN significantly down-regulated aggrecanase-1 and MMP3 and 5 mM GlcN-S additionally down-regulated aggrecanase-2 and tissue inhibitor of MMP gene expression significantly. Gene expression was not significantly altered by 0.5 mM GlcN. Total MMP activity in culture medium was only significantly reduced after addition of 5 mM GlcN-HCl.

Conclusion

The effects of GlcN on gene expression in a human osteoarthritic explant model suggest that enzymatic breakdown of the extracellular matrix might be reduced by the addition of 5 mM GlcN. Additionally, restoration of already damaged cartilage is not to be expected, because gene expression of anabolic genes is also down-regulated. We suggest that chondroprotective properties of GlcN *in vivo*, may be based on inhibiting further degradation due to catabolic activities, rather than on the ability to rebuild cartilage.

INTRODUCTION

Glucosamine (GlcN) is becoming increasingly popular as an alternative treatment for osteoarthritis (OA). There is evidence in the literature that GlcN is equally effective or even better in decreasing pain in patients with knee OA, as compared to low dose Non-Steroidal Anti-Inflammatory Drugs (NSAID) use [1,2]. Furthermore, two publications showed less joint space narrowing in people with knee OA who took GlcN compared to placebo, over a period of 3 years [3,4]. Both articles conclude that GlcN could delay the progression of knee OA. The authors speculate that GlcN might have an effect on the chondrocytes due to stimulation of anabolic activities and depression of catabolic activities. Some *in vitro* studies indeed showed a positive effect of GlcN on glycosaminoglycan (GAG) production in human chondrocytes cell cultures and the same anabolic effect was found on bovine and rat explants [5-9]. Controversially, others found no effect or even an inhibition on GAG production following GlcN addition [10-16]. Inconsistent results were also found for the effect of GlcN on catabolic activities. These *in vitro* studies used addition of interleukin-1 (IL-1), lipopolysaccharide or retinoic acid to mimic a degenerative environment for chondrocytes in culture and thereby induce the synthesis of catabolic factors such as matrix metalloproteinase (MMP) and aggrecanase. Considering the final result of catabolic activities, i.e., degradation of GAG, studies using IL-1 as a model for degeneration found that GlcN decreased GAG degradation in some cases, but in other cases no such effect was found [6,11,12,17,18]. In the studies using lipopolysaccharide or retinoic acid as a model for degeneration, treatment with GlcN consistently led to a decreased GAG degradation in all cases [12,14,17,19-21]. Since GAG degradation finally is the result of enzymatic breakdown, several studies investigated the effects of GlcN on extracellular matrix degrading enzymes in a culture system using IL-1, lipopolysaccharide or retinoic acid. Most of these studies found that addition of GlcN led to less MMP activity and aggrecanase activity [6,11,16,18,20,22]. One study observed no effect on MMP activity and another study found no changes in aggrecanase activity after GlcN addition [14,21].

The majority of the *in vitro* studies testing GlcN have been performed with chondrocytes from different animal species (bovine, equine, rat, dog and mouse chondrocytes). We found only eight studies in the literature that used human chondrocytes and none of these studies used the physiologically relevant human osteoarthritic cartilage explant culture system [5-7,10,23-27].

The aim of our study was to investigate the expression of genes, involved in both anabolic and catabolic activities of chondrocytes, in response to GlcN treatment in a human OA explant model. In addition to gene expression screening we also studied whether changes in the transcription led to altered overall enzymatic activity as well.

MATERIALS AND METHODS

Explant preparation

Human osteoarthritic cartilage was obtained during total knee replacement surgery (12 patients; age 51-79 years, Kellgren and Lawrence grade 2.9 ± 0.67 (mean ± SD)). Pre-operative treatment regimes were not considered. For each patient, the experimental condition was compared to the control condition. Explants were taken from areas of macroscopically normal cartilage (with knowledge that this cartilage is affected by the disease process) from both the femoral condyles and the tibial plateau using a 4 mm diameter dermal biopsy punch, and freed from the underlying bone by dissection with a scalpel. After dissection the explants were all pooled in a Petri dish. For each condition, six explants were randomly taken from the Petri dish and cultured in a six-well plate with 3 ml low glucose (1000 mg/l, 5.55 mM) Dulbecco's Modified Eagle Medium (D-MEM; Gibco, Grand Island, NY), supplemented with 10% foetal calf serum (FCS), 50 µg/ml gentamicin, 1.5 µg/ml fungizone and freshly added 25 µg/ml L-ascorbic acid-2-phosphate, during the whole experiment. The amount of glucose present in the FCS added was less than 0.5 mM and is neglected. We only started an explant culture when the amount of cartilage was enough to make at least 12 explants and we were thus able to culture experimental and one control conditions.

Culture experiments

After an initial 3-day pre-culture period (day 0-3), experimental reagents were added for 4 days (day 3-7). For the experimental conditions, culture medium (see above) was supplemented with 0.5 mM and 5 mM glucosamine hydrochloride (GlcN-HCl; Sigma, St Louis, MO) or glucosamine-sulphate (GlcN-S; Sigma, St Louis, MO). As a control, culture medium and culture medium supplemented with 5 mM glucose (Gluc; Sigma, St Louis, MO; final Gluc concentration 10.5 mM) were used. Culture medium with or without supplements was refreshed once. Conditioned medium from all time points of refreshment and when the explants were harvested was stored at –20°C for analysis. Explants were harvested after a total of 7 days of culture.

Gene expression analysis

At harvesting, six explants were collected and snap frozen in liquid nitrogen. The wet weight per sample was determined and the frozen cartilage was then processed using the Mikro-Dismembrator S® (B. Braun Biotech International GmbH, Melsungen, Germany). RNA was extracted using RNA-Bee™ (TEL-TEST, Inc; Friendswood, TX, USA) according to manufacturer's guidelines and subsequently precipitated with 2-propanol. RNA was further purified using RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) with on-column DNA-digestion. Total RNA was quantified accurately using Ribogreen™ reagent (R-11490, Molecular Probes Europe BV, Leiden, The Netherlands) according to manufacturer's instructions and 500 ng total RNA of each sample was reverse transcribed into complementary DNA (cDNA) using RevertAid™ First

Table 1. Primer and probe nucleotide sequences of the tested genes

	Accession No.	Primer		Probe
COL2	NM_033150	Fw:	GGCAATAGCAGGTTCACGTACA	CCGGTATGTTTCGTGCAGCCATCCT
		Rv:	CGATAACAGTCTTGCCCCACTT	
AGC1	NM_001135	Fw:	TCGAGGACAGCGAGGCC	ATGGAACACGATGCCTTTCACCACGA
		Rv:	TCGAGGGTGTAGCGTGTAGAGA	
MMP1	NM_002421	Fw:	CTCAATTTCACTTCTGTTTTCTG	CACAACTGCCAAATGGGCTTGAAGC
		Rv:	CATCTCTGTCGGCAAATTCGT	
MMP2	NM_004530	Fw:	TCAAGTTCCCCGGCGAT	TCGCCCCCAAAACGGACAAAGA
		Rv:	TGTTCAGGTATTGCACTGCCA	
MMP3	NM_002422	Fw:	TTTTGGCCATCTCTTCCTTCA	AACTTCATATGCGGCATCCACGCC
		Rv:	TGTGGATGCCTCTTGGGTATC	
MMP9	NM_004994	Fw:	TGAGAACCAATCTCACCGACAG	CAGCTGGCAGAGGAATACCTGTACCGC
		Rv:	TGCCACCCGAGTGTAACCAT	
MMP13	NM_002427	Fw:	AAGGAGCATGGCGACTTCT	CCCTCTGGCCTGCTGGCTCA
		Rv:	TGGCCCAGGAGGAAAAGC	
MMP14	NM_004995	Fw:	TGCCTGCGTCCATCAACACT	AAGACGAATTTGCCATCCTTCCTCTCGT
		Rv:	CATCAAACACCCAATGCTTGTC	
TIMP1	NM_003254	Fw:	TGCCGCATCGCCGAGAT	CCAGCGCCCAGAGAGAC
		Rv:	ATGGTGGGTTCTCTGGTG	
TIMP2	NM_003255	Fw:	ATGGTGGGTTCTCTGGTG	CCTGCATCAAGAGAAGTGAC
		Rv:	CGGTACCACGCACAGGA	
TIMP3	NM_000362	Fw:	AGGACACATTTTGCCCGATG	CCACCCCCAGGACGCCTTCTG
		Rv:	TGCACATGCTCGCCCA	
ADAMTS1	NM_006988	Fw:	GGACAGGTGCAAGCTCATCTG	CAAGCCAAAGGCATTGGCTACTTCTTCG
		Rv:	TCTACAACCTTGGGCTGCAAA	
ADAMTS4	NM_005099	Fw:	CAAGGTCCCATGTGCAACGT	CCGAAGAGCCAAGCGCTTTGCTTC
		Rv:	CATCTGCCACCACCAGTGTCT	
ADAMTS5	NM_007038	Fw:	TGTCCTGCCAGCGGATGT	TTCTCCAAAGGTGACCGATGGCACTG
		Rv:	ACGGAATTACTGTACGGCCTACA	

Fw: forward; Rv: reverse

Strand cDNA Synthesis Kit (MBI Fermentas, Germany). Primers and probe sets were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet TaqMan® requirements and were designed to bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. BLASTN search was used to ensure gene specificity of all oligo-nucleotide sequences. The primer and probe nucleotide sequences can be found in Table 1. Collagen II (COL2) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay were adopted from Martin et al. [28]. Both anabolic genes (encoding for extracellular matrix components) and catabolic genes (involved in degradation of the extracellular matrix) were studied. TaqMan® assays were performed on an ABI 7700 as described earlier and data are presented as relative expression normalized to GAPDH ($2^{-\Delta\Delta Ct}$) according to Mandl et al. [29] and Livak and Schmittgen [30].

Total MMP assay

The stored culture medium was used to determine general MMP activity as described earlier [31]. General MMP activity was measured using 5 μM (all concentrations are final) fluorogenic substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH_2) in the presence or absence of 12.5 μM BB94 (a general MMP inhibitor). Medium samples were diluted (final dilution 1/2) in MMP buffer (50 mM Tris [pH 7.5], 5 mM $CaCl_2$, 150 mM NaCl, 1 μM $ZnCl_2$, 0.01% Brij-35, 0.02% NaN_3) containing the general proteinase inhibitor (Complete EDTA-free, one tablet in 10 ml). The MMP activity in each sample was calculated as the difference in the initial rate of substrate conversion (linear increase in fluorescence in time, expressed as relative fluorescence units per second) between samples with and without BB94 addition. Fluorescence was measured for 6 h at 30°C using a Cytofluor 4000 (Applied Biosystems, Foster City, CA, USA). This assay is considered to represent overall MMP activity.

Data analyses

From the material obtained from each patient, one control culture and at least one experimental culture were performed. The Ct-values of each control and experimental condition were normalized to GAPDH. Hereafter each experimental condition was expressed relative to the corresponding control condition of the same patient, according to the $2^{-\Delta\Delta Ct}$ method. The resulting number of this calculation indicates whether there is an up- or down-regulation of gene expression in an experimental condition compared to its paired untreated control. Now for each subset of experimental conditions (e.g., GlcN-HCl 5 mM, GlcN-S 0.5 mM) the median of these numbers was calculated. For graphical display purposes, only the $2^{-\Delta\Delta Ct}$ values were expressed as a ^{10}LOG. Because of the sampling size we used box-whisker plots, with the box representing the middle two quartiles (25-75) and the whiskers the highest and lowest values, with exclusion in case of outlier variables. Total MMP activity was calculated as relative fluorescence units per second and presented as mean and standard deviation relative to the untreated control, which was set at 100%.For statistical analysis a Friedman test with post-hoc Wilcoxon signed ranks test was performed on the normalized Ct values (gene expression) and the relative fluorescence units per second (total MMP activity), using SPSS 11.0.1 (SPSS Inc., Chicago, IL). A p-value ≤ 0.05 was considered to indicate statistically significant differences.

RESULTS

Anabolic activities

Collagen type II expression (Fig. 1) was down-regulated by addition of 5 mM GlcN-HCl (7.75-fold; p=0.005, N=10) and by 5 mM GlcN-S (22.17-fold; p=0.005, N=10). Aggrecan gene expression (Fig. 2) decreased 2.65-fold by addition of 5mM GlcN-HCl (p=0.012, N=8) and 7.73-fold by 5 mM GlcN-S (p=0.008, N=9). Gene expression was not significantly altered by addition of 0.5 mM

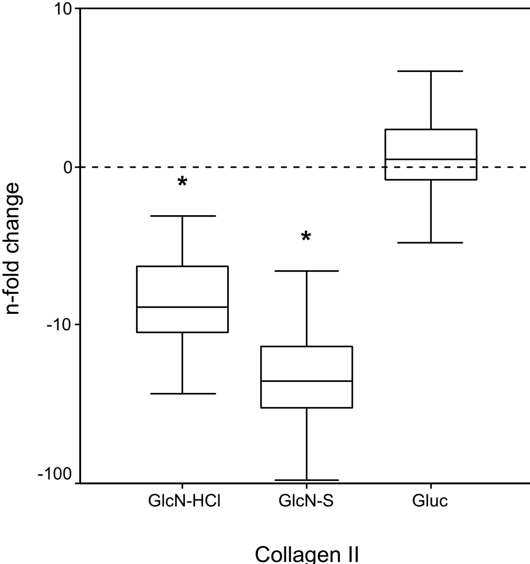

Figure 1. Change in collagen type II gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl (N=10), 5 mM GlcN-S (N=10) or 5 mM Gluc (N=9). The n-fold change, normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is displayed on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression.
* indicates a p-value ≤ 0.05.

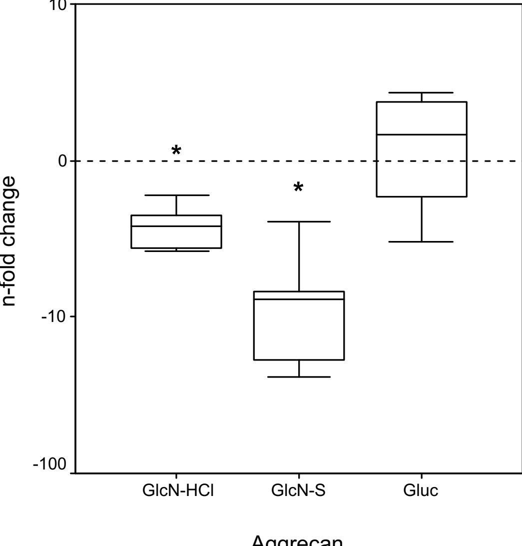

Figure 2. Change in aggrecan gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl (N=8), 5 mM GlcN-S (N=9) or 5 mM Gluc (N=7). The n-fold change, normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is shown on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. * indicates a p-value ≤ 0.05.

GlcN-HCl or 0.5 mM GlcN-S, but showed a trend similar to the 5 mM concentration. No effect of addition of 5 mM Gluc was found.

Catabolic activities

ADAMTS1 (Fig. 3 A) showed no significant alteration in gene expression for both GlcN derivatives (N=10). Aggrecanase-1 (ADAMTS4) (Fig. 3 B) was down-regulated 6.38-fold by 5 mM GlcN-HCl (p=0.005, N=10) and 7.83-fold by 5 mM GlcN-S (p=0.005, N=10). Aggrecanase-2 (ADAMTS5) (Fig. 3 C) revealed a significant down-regulation only upon addition of 5 mM GlcN-S (median 4.51-fold; p=0.005, N=10).

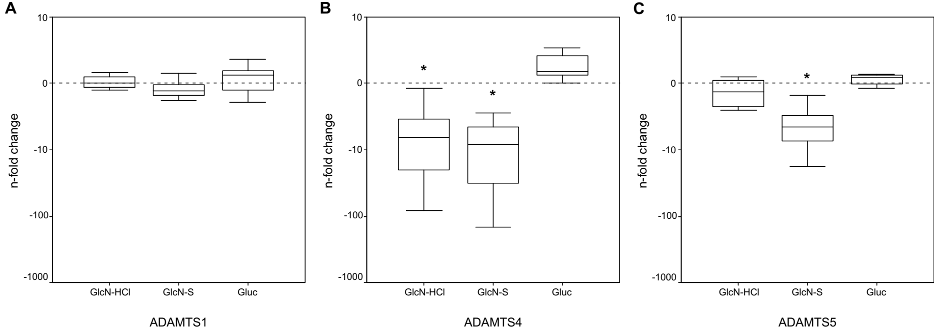

Figure 3. Change in aggrecanase gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl (N=10), 5 mM GlcN-S (N=10) or 5 mM Gluc (N=9).The n-fold change for ADAMTS1 (panel A), ADAMTS4 (panel B) and ADAMTS5 (panel C), normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is displayed on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. * indicates a p-value ≤ 0.05.

GlcN had no statistically significant effect on the expression of the majority of the MMP genes that were tested (i.e., MMP1, 2, 9, 13 and 14). MMP3 (Fig. 4) was the only MMP that showed a significant down-regulation of gene expression in response to both 5 mM GlcN-HCl (2.02-fold; p=0.005, N=10) and 5 mM GlcN-S (2.66-fold; p=0.005, N=10).

The MMP activity assay (Fig. 5) showed an activity for 5mM GlcN-HCl of 79.5 ± 15.6% (mean ± SD, N=10) and 91.1 ± 17.1% (mean ± SD, N=10) for 5 mM GlcN-S, as compared to the total MMP activity of the untreated control which was set at 100%. This reduction in total MMP activity was only statistically significant for GlcN-HCl (p=0.047). For both 0.5 mM concentrations of the GlcN derivatives total MMP activity was not reduced as compared to the untreated control.

In order to obtain a broad view of the catabolic potential, expression of the natural tissue inhibitors of MMPs (TIMPs) was also studied. The gene expression of the TIMPs (Fig. 6) was only slightly down-regulated by the addition of 5 mM GlcN-HCl and 5 mMGlcN-S. The only statistically significant alteration was a 3.07-fold down-regulation of TIMP3 gene expression after addition of 5 mM GlcN-S (N=10). Gene expression was not significantly altered by addition of

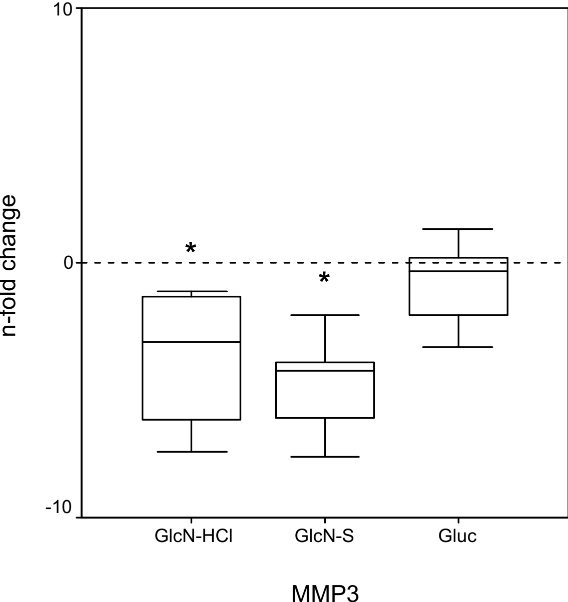

Figure 4. Change in MMP3 gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl (N=10), 5 mM GlcN-S (N=10) or 5 mM Gluc (N=9). The n-fold change, normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is shown on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. * indicates a p-value ≤ 0.05.

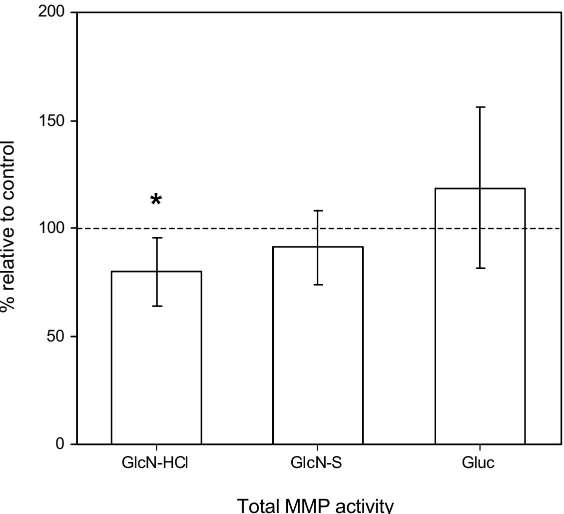

Figure 5. Total MMP activity in the culture medium of human osteoarthritic cartilage explants after 3 days pre-culture, followed by 4 days of treatment with 5 mM GlcN-HCl (N=10), 5 mM GlcN-S (N=10) or 5 mM Gluc (N=9). Total MMP activity is displayed as a percentage relative to the MMP activity in medium of the untreated control, which was set at 100% (indicated by the dotted line). * indicates a p-value ≤ 0.05.

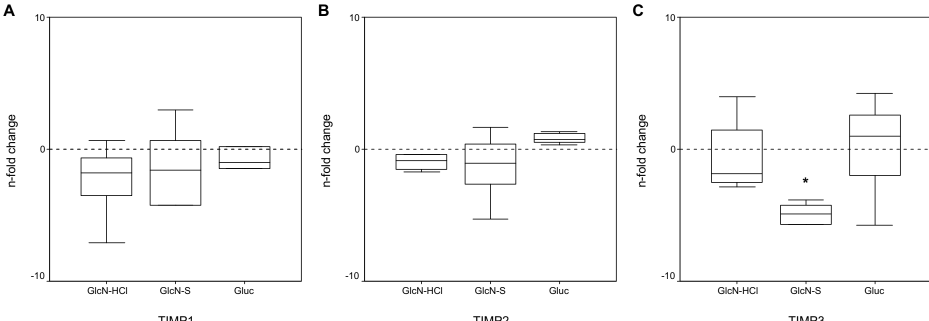

Figure 6. Change in TIMP gene expression in human osteoarthritic cartilage after culture with GlcN. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 5 mM GlcN-HCl, 5 mM GlcN-S or 5 mM Gluc. The n-fold change for TIMP1 (panel A; GlcN-HCl N=10, GlcN-S N=10, Gluc N=9), TIMP2 (panel B; GlcN-HCl N=9, GlcN-S N=9, Gluc N=8) and TIMP3 (panel C; GlcN-HCl N=10, GlcN-S N=10, Gluc N=9), normalized to GAPDH and relative to the untreated control (indicated by the dotted line) is shown on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. * indicates a p-value ≤ 0.05. Missing whiskers in a graph are due to the exclusion of outliers, which results in the 25th and 75th percentile becoming the lowest and the highest value, respectively.

0.5 mM GlcN-HCl or 0.5 mM GlcN-S, but showed a trend similar to the 5 mM concentration. No effect of addition of 5 mM Gluc was found.

Catabolic gene expression was not substantially influenced by 5 mM Gluc, with the exception of a 1.54-fold up-regulation of the MMP14 gene expression compared to control (p = 0.021, N=9).

GlcN-HCl versus GlcN-S

To explain some of the literature differences regarding the *in vitro* effects of GlcN, we also tested if there was a difference in alteration of gene expression between treatment with 5 mM GlcN-HCl and 5 mM GlcN-S. We found that expression of ADAMTS5 (p= 0.008, N=9), TIMP3 (p= 0.008, N=9) and aggrecan (p= 0.012, N=8) was significantly more down-regulated by 5 mM GlcN-S than by 5 mM GlcN-HCl.

DISCUSSION

Addition of GlcN derivatives (both the sulphate and the hydrochloride salt) at a concentration of 5 mM to osteoarthritic cartilage *in vitro* leads to a down-regulation of genes that encode for anabolic (e.g., COL2 and aggrecan) and catabolic (ADAMTS enzymes and MMPs) processes. For the MMPs, this was also shown at protein activity level.

From all published studies that found an inhibition of proteoglycan production by the addition of GlcN, four were performed using an cartilage explant model [12,14,17,19]. Two of these studies used equine cartilage and the other two used bovine cartilage. In all these studies, IL-1,

lipopolysaccharide or retinoic acid was used to induce a catabolic response and mimic OA. The GlcN concentrations that significantly reduced proteoglycan production varied from 5 mM to 116 mM. Lower GlcN concentrations used in these studies did not lead to a significant decrease of proteoglycan production. This is in agreement with our finding that 5 mM concentrations of both GlcN derivatives led to a significant down-regulation of aggrecan gene expression, while the 0.5 mM concentrations did not. Although it was not a subject of our investigation, it might be expected that a down-regulation of aggrecan gene expression as we have found ultimately will lead to less GAG production.

Two earlier studies using human cell clusters found no effect on COL2 production after addition of crystalline GlcN-S (Dona®) at concentrations up to 0.56 mM [5,7]. In our data we could not find a significant effect for this concentration as well. In contrast, for the 5 mM concentration we found a significant down-regulation of COL2 gene expression.

In osteoarthritic cartilage, expression of MMPs, especially, MMP1, 2, 3, 9, 13 and 14, has been demonstrated [32]. In the present study we show that addition of GlcN to culture medium inhibits MMP activity. In the assay we used, only active MMPs were measured. While pro-MMP levels only provide information on the potential of the system to breakdown matrix, active MMPs are the molecular forms of the enzyme that causes the actual tissue breakdown. On gene expression level, only MMP3 (stromelysin-1) was significantly down-regulated. At this point we do not have an explanation for this. We can speculate that it probably has to do with differences in regulation of gene expression of the different MMPs. In our view, this apparent rather selective inhibition of MMP3 may even be an advantage. If GlcN would down-regulate all MMPs (as if it were a broad-spectrum MMP inhibitor) normal tissue turnover in other connective tissues than cartilage may also be suppressed, causing unwanted side effects. The apparent selective MMP3 inhibition may block the excess proteolytic activity that is inherent to the OA disease state, due to its pivotal role in the MMP activation cascade [33], but may leave normal tissue turnover unaffected. We have found that addition of 5 mM GlcN-HCl or 5 mM GlcN-S leads to a significant down-regulation, when compared to the untreated control. This significant down-regulation is in accordance with the findings by Gouze et al.[16], who used rat chondrocytes in monolayer culture stimulated with IL-1β to mimic OA. The addition of GlcN in the same concentration as Gluc to the culture medium, led to significant lower MMP3 mRNA levels. In contrast, another study using human osteoarthritic cells in cell suspension culture, showed no effect on MMP3 mRNA levels with GlcN-S concentration up to 0.2 mM [6]. The latter being in agreement with our study, as we could not find a significant effect of the 0.5 mM concentrations on MMP3 gene expression either. Lower MMP activity in the culture medium as we observed upon the addition of 5 mM GlcN, was also described in 2 studies in which GlcN was added to lipopolysaccharide treated equine chondrocytes [17,22]. Thus, our results suggest that addition of GlcN *in vitro* leads to less MMP-mediated extracellular matrix degradation.

Next to MMP3, our results showed that ADAMTS4 expression is down-regulated by GlcN-HCl and GlcN-S and that ADAMTS5 expression is significantly down-regulated by 5 mM GlcN-S. This confirms the results of Sandy et al.[11] who found less aggrecanase activity in a rat chondrosarcoma cell line and bovine explants after addition of GlcN in a dose dependent manner. This down-regulation of ADAMTS4 and ADAMTS5 by addition of GlcN might lead to preservation of the extracellular matrix, since these two aggrecanases are significantly up-regulated in OA, when compared to normal cartilage [34]. Addition of GlcN had no effect on expression of ADAMTS1, which was also identified as an aggrecanase [35]. Since up-regulation of ADAMTS1 expression has not been reported in OA, its aggrecan-degrading activity seems of less importance [36].

To obtain a broad view on MMP activation, the expression of TIMPs was studied. TIMP1, 2 and 3 are important regulators of the proteolytic activity of MMPs by endogenous inhibition [37]. Addition of GlcN did not affect the expression of TIMP1 and TIMP2. Apart from inhibitory effects on MMPs, TIMP3 was also shown to be a potent endogenous inhibitor of aggrecanases and therefore protects against aggrecanase-mediated cartilage degeneration[38-40]. Since TIMP3 was significantly down-regulated by 5 mM GlcN-S, aggrecanase activity might be less inhibited as expected purely based on the down-regulation of ADAMTS4 and ADAMTS5 gene expression upon adding 5 mM GlcN-S *in vitro*.

To evaluate cell death related effects, due to toxicity of the used GlcN concentration in our study, we compared the total amount of RNA per milligram wet weight of the original tissue at the end of the culture period between control group and 5 mM GlcN treated conditions. No statistically significant difference was found. Furthermore, we found no significant difference in GAPDH expression between control group and all tested 5 mM concentrations. We thus believe that the effects we have found on gene expression are not based on cell death, but on actual regulatory effects of GlcN. Detrimental effects of high dose GlcN-HCl on bovine cartilage explants have been reported, but only with concentrations above 10 mM, which were twice as high as was used in this study [12]. The same group did not observe any effects on cell viability after 24 h culture with less than 10 mM GlcN-HCl.

In osteoarthritic knee-joint effusions, the mean Gluc concentration was previously shown to be 5.4 mM, with a range comparable to the reference range for serum [41]. With this in mind we decided to use medium with low glucose concentration (5.55 mM), since this comes closest to the physiological situation. In the experimental conditions, GlcN was added at concentrations equimolar with or 10 times lower than glucose concentration in medium. *In vitro*, exogenous GlcN was shown to be incorporated in newly formed chondroitin sulphate in cultured mouse chondrocytes and immortalized human chondrocytes when added at an equimolar concentration with Gluc in the culture medium [10,42]. When Gluc concentration became higher than the GlcN concentration, cells utilized less exogenous GlcN for the formation of chondroitin sulphate, but preferably incorporated GlcN that was endogenously formed from Gluc. These results suggest that not only the absolute concentration of GlcN, but also the GlcN-to-Gluc ratio

plays an important role in the utilization and therefore effectiveness of exogenously provided GlcN. This might explain why we did not find any significant results of the addition of 0.5 mM GlcN in culture medium with a 10 times higher glucose concentration.

When trying to translate our *in vitro* results to clinical *in vivo* applicability of GlcN, the intra-articular GlcN concentration that can be reached after administration of this food additive to the patient is of concern. Since the first studies were performed on the effect of GlcN on articular cartilage there has been debate on this topic. When GlcN-HCl is administered to horses in a single dose, intravenously as well as orally, in a dosage per kg bodyweight at clinically relevant levels, GlcN concentrations in the synovial fluid ranged from 9 μM to 15 μM and from 0.3 μM to 0.7 μM, respectively [43]. Although these concentrations were less than 10% of the obtained serum concentrations at the same time, GlcN was still detectable in synovial fluids 6 hours after it was nearly completely cleared from the serum. Several studies with radioactive labelled GlcN administered to animals have shown that articular cartilage has the capacity to accumulate and retain GlcN [44-46]. This is confirmed in a study with six healthy male volunteers who received a single dose ^{14}C labelled GlcN-S orally, intravenously or intramuscularly [47]. These studies indicate that due to its special capacity to accumulate and retain GlcN, the concentrations of GlcN within the articular cartilage can actually be much higher than those found in the surrounding synovial fluid. It might however still be questioned whether 5 mM levels will ever reach the joint. In contrast with the previously mentioned synovial glucose concentration of 5 mM, Windhaber et al. [48] mentioned, based on measurements with microelectrodes, a Gluc concentration of 1 mM directly surrounding the chondrocytes. With the possible importance of the GlcN-to-Gluc ratio in mind, GlcN concentration perhaps does not have to be as high as 5 mM, in order make exogenous GlcN effective *in vivo*.

In conclusion, our results suggest that enzymatic breakdown of the extracellular matrix *in vitro* might be reduced by the addition of GlcN. This was suggested by down-regulation of transcript abundances and reduced MMP enzymatic activity, which preserves the cartilage matrix in the catabolic OA situation. On the other hand, on transcription level we showed that treatment with 5 mM GlcN-HCl or 5 mM GlcN-S led to a significant down-regulation of collagen type II and aggrecan expression. Whether this down-regulation of gene expression also results in less extracellular matrix production was not investigated in this study. However, restoration of already damaged cartilage is not to be expected. Taking this into consideration, our results indicate that chondroprotective properties of GlcN may be based on inhibiting further degradation due to catabolic activities, rather than on the ability to rebuild cartilage.

REFERENCES

1. Muller-Fassbender H, Bach GL, Haase W, Rovati LC, Setnikar I. Glucosamine sulfate compared to ibuprofen in osteoarthritis of the knee. Osteoarthritis Cartilage 1994;2:61-9.
2. Lopes Vaz A. Double-blind clinical evaluation of the relative efficacy of ibuprofen and glucosamine sulphate in the management of osteoarthrosis of the knee in out-patients. Curr Med Res Opin 1982; 8:145-9.
3. Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002;162:2113-23.
4. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, *et al*. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-6.
5. Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-34.
6. Dodge GR, Jimenez SA. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis Cartilage 2003;11:424-32.
7. Bassleer C, Henrotin Y, Franchimont P. In-vitro evaluation of drugs proposed as chondroprotective agents. Int J Tissue React 1992;14:231-41.
8. Lippiello L. Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress. Osteoarthritis Cartilage 2003;11:335-42.
9. Vidal y Plana RR, Bizzarri D, Rovati AL. Articular cartilage pharmacology: I. In vitro studies on glucosamine and non steroidal antiinflammatory drugs. Pharmacol Res Commun 1978;10:557-69.
10. Mroz PJ, Silbert JE. Use of (3)H-glucosamine and (35)S-sulfate with cultured human chondrocytes to determine the effect of glucosamine concentration on formation of chondroitin sulfate. Arthritis Rheum 2004;50:3574-9.
11. Sandy JD, Gamett D, Thompson V, Verscharen C. Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. Biochem J 1998;335 (Pt 1):59-66.
12. de Mattei M, Pellati A, Pasello M, de Terlizzi F, Massari L, Gemmati D, *et al*. High doses of glucosamine-HCl have detrimental effects on bovine articular cartilage explants cultured in vitro. Osteoarthritis Cartilage 2002;10:816-25.
13. Anderson CC, Cook JL, Kreeger JM, Tomlinson JL, Wagner-Mann CC. In vitro effects of glucosamine and acetylsalicylate on canine chondrocytes in three-dimensional culture. Am J Vet Res 1999;60: 1546-51.
14. Ilic MZ, Martinac B, Handley CJ. Effects of long-term exposure to glucosamine and mannosamine on aggrecan degradation in articular cartilage. Osteoarthritis Cartilage 2003;11:613-22.
15. Gouze JN, Bianchi A, Becuwe P, Dauca M, Netter P, Magdalou J, *et al*. Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-kappa B pathway. FEBS Lett 2002;510:166-70.
16. Gouze JN, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, *et al*. Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis: influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. Arthritis Rheum 2001;44:351-60.
17. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:258-65.
18. Fenton JI, Chlebek-Brown KA, Caron JP, Orth MW. Effect of glucosamine on interleukin-1-conditioned articular cartilage. Equine Vet J Suppl 2002:219-23.

19. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:444-51.
20. Orth MW, Peters TL, Hawkins JN. Inhibition of articular cartilage degradation by glucosamine-HCl and chondroitin sulphate. Equine Vet J Suppl 2002:224-9.
21. Mello DM, Nielsen BD, Peters TL, Caron JP, Orth MW. Comparison of inhibitory effects of glucosamine and mannosamine on bovine articular cartilage degradation in vitro. Am J Vet Res 2004;65:1440-5.
22. Byron CR, Orth MW, Venta PJ, Lloyd JW, Caron JP. Influence of glucosamine on matrix metalloproteinase expression and activity in lipopolysaccharide-stimulated equine chondrocytes. Am J Vet Res 2003;64:666-71.
23. Shikhman AR, Kuhn K, Alaaeddine N, Lotz M. N-acetylglucosamine prevents IL-1 beta-mediated activation of human chondrocytes. J Immunol 2001;166:5155-60.
24. Nakamura H, Shibakawa A, Tanaka M, Kato T, Nishioka K. Effects of glucosamine hydrochloride on the production of prostaglandin E2, nitric oxide and metalloproteases by chondrocytes and synoviocytes in osteoarthritis. Clin Exp Rheumatol 2004;22:293-9.
25. Largo R, Alvarez-Soria MA, Diez-Ortego I, Calvo E, Sanchez-Pernaute O, Egido J, *et al.* Glucosamine inhibits IL-1beta-induced NFkappaB activation in human osteoarthritic chondrocytes. Osteoarthritis Cartilage 2003;11:290-8.
26. Piperno M, Reboul P, Hellio Le Graverand MP, Peschard MJ, Annefeld M, Richard M, *et al.* Glucosamine sulfate modulates dysregulated activities of human osteoarthritic chondrocytes in vitro. Osteoarthritis Cartilage 2000;8:207-12.
27. Lafeber FP, Vander Kraan PM, Van Roy JL, Huber-Bruning O, Bijlsma JW. Articular cartilage explant culture; an appropriate in vitro system to compare osteoarthritic and normal human cartilage. Connect Tissue Res 1993;29:287-99.
28. Martin I, Jakob M, Schafer D, Dick W, Spagnoli G, Heberer M. Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. Osteoarthritis Cartilage 2001;9: 112-8.
29. Mandl EW, Jahr H, Koevoet JL, van Leeuwen JP, Weinans H, Verhaar JA, *et al.* Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture. Matrix Biol 2004;23:231-41.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:40231. DeGroot J, Verzijl N, Wenting-Van Wijk MJ, Bank RA, Lafeber FP, Bijlsma JW, *et al.* Age-related decrease in susceptibility of human articular cartilage to matrix metalloproteinase-mediated degradation: the role of advanced glycation end products. Arthritis Rheum 2001;44:2562-71.
32. Bramono DS, Richmond JC, Weitzel PP, Kaplan DL, Altman GH. Matrix Metalloproteinases and Their Clinical Applications in Orthopaedics. Clin Orthop 2004;1:272-85.
33. Somerville RP, Oblander SA, Apte SS. Matrix metalloproteinases: old dogs with new tricks. Genome Biol 2003;4:216.
34. Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E, Aigner T. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. Arthritis Rheum 2002;46:2648-57.
35. Nagase H, Kashiwagi M. Aggrecanases and cartilage matrix degradation. Arthritis Res Ther 2003;5: 94-103.
36. Wachsmuth L, Bau B, Fan Z, Pecht A, Gerwin N, Aigner T. ADAMTS-1, a gene product of articular chondrocytes in vivo and in vitro, is downregulated by interleukin 1beta. J Rheumatol 2004;31:315-20.
37. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. J Biol Chem 1999;274:21491-4.
38. Hashimoto G, Aoki T, Nakamura H, Tanzawa K, Okada Y. Inhibition of ADAMTS4 (aggrecanase-1) by tissue inhibitors of metalloproteinases (TIMP-1, 2, 3 and 4). FEBS Lett 2001;494:192-5.
39. Kashiwagi M, Tortorella M, Nagase H, Brew K. TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). J Biol Chem 2001;276:12501-4.

40. Gendron C, Kashiwagi M, Hughes C, Caterson B, Nagase H. TIMP-3 inhibits aggrecanase-mediated glycosaminoglycan release from cartilage explants stimulated by catabolic factors. FEBS Lett 2003; 555:431-6.
41. Krachler M, Domej W. Clinical laboratory parameters in osteoarthritic knee-joint effusions correlated to trace element concentrations. Biol Trace Elem Res 2001;79:139-48.
42. Mroz PJ, Silbert JE. Effects of [3H]glucosamine concentration on [3H]chondroitin sulphate formation by cultured chondrocytes. Biochem J 2003;376:511-5.
43. Laverty S, Sandy JD, Celeste C, Vachon P, Marier JF, Plaas AH. Synovial fluid levels and serum pharmacokinetics in a large animal model following treatment with oral glucosamine at clinically relevant doses. Arthritis Rheum 2005;52:181-91.
44. Setnikar I, Giacchetti C, Zanolo G. Pharmacokinetics of glucosamine in the dog and in man. Arzneimittelforschung 1986;36:729-35.
45. Setnikar I, Giachetti C, Zanolo G. Absorption, distribution and excretion of radioactivity after a single intravenous or oral administration of [14C] glucosamine to the rat. Pharmatherapeutica 1984;3:538-50.
46. Setnikar I, Rovati LC. Absorption, distribution, metabolism and excretion of glucosamine sulfate. A review. Arzneimittelforschung 2001;51:699-725.
47. Setnikar I, Palumbo R, Canali S, Zanolo G. Pharmacokinetics of glucosamine in man. Arzneimittelforschung 1993;43:1109-13.
48. Windhaber RA, Wilkins RJ, Meredith D. Functional characterisation of glucose transport in bovine articular chondrocytes. Pflugers Arch 2003;446:572-7.

Chapter 3

Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate

E.J. Uitterlinden, H. Jahr, J.L.M. Koevoet, S.M.A. Bierma-Zeinstra, J.A.N. Verhaar, H. Weinans, G.J.V.M. van Osch

Osteoarthritis & Cartilage 2007 Nov; 15(11):1267-74

ABSTRACT

Objective

To investigate the working mechanism of glucosamine (GlcN) by studying the effect of different GlcN derivatives on bovine chondrocytes in alginate beads under anabolic and catabolic culture conditions.

Methods

Bovine chondrocytes seeded in alginate beads were treated with different concentrations of glucosamine-sulphate (GlcN-S), glucosamine-hydrochloride (GlcN-HCl) or N-acetyl-glucosamine (GlcN-Ac). Culture conditions were anabolic, 3 day pre-culture followed by 14 days' treatment; catabolic, extracellular matrix (ECM) breakdown induced by 10 ng/ml interleukin-1β (IL-1β); or a situation with balance between ECM breakdown and synthesis, 24 days' pre-culture followed by 14 days' treatment. The outcome measurements were total glycosaminoglycan (GAG) and DNA content per bead.

Results

In the situation with balance between ECM breakdown and synthesis, GlcN-Ac had a small stimulatory effect on total GAG content. GlcN-S and GlcN-HCl had no effect. Under anabolic condition 5 mM GlcN-S and GlcN-HCl significantly reduced total GAG content. GlcN-Ac did not show this effect. IL-1β induced catabolic effects were prevented by adding 5 mM GlcN-HCl. Interference of GlcN with glucose (Gluc) was demonstrated by adding extra Gluc to the medium in the anabolic culture conditions. Increasing extracellular Gluc concentrations diminished the effect of GlcN.

Conclusion

GlcN-S and GlcN-HCl, but not GlcN-Ac, reduce anabolic and catabolic processes. For anabolic processes this was demonstrated by decreased ECM synthesis, for catabolic processes by protection against IL-1β mediated ECM breakdown. This might be due to interference of GlcN with Gluc utilization. We suggest that the claimed structure modifying effects of GlcN are more likely based on protection against ECM degradation than new ECM production.

INTRODUCTION

Nutraceuticals, in particular glucosamine (GlcN), are increasingly used as a treatment option for osteoarthritis (OA). Over the last decades an ever increasing number of patients have started using GlcN, with or without a prescription of their physician. Although the effectiveness of GlcN has been debated in a recent article reporting a large NIH trial, subgroup analysis indicated that there might be an effect in people with mild to severe OA [1]. Other clinical studies suggested that GlcN probably has structure modifying effects in patients with knee OA [2,3]. The underlying effects of GlcN on cartilage, responsible for these clinical outcomes, are still not clear. Conflicting results on this topic in *in vitro* experiments are reported in the literature. It has been proposed that addition of GlcN to chondrocyte cell cultures leads to more glycosaminoglycan (GAG) production, since GlcN is the basic building block of GAG molecules. Some studies showed results in favor of this hypothesis [4-8]. But, other studies could not confirm this effect [9,10]. And furthermore there are studies that observed negative effects on GAG production after GlcN addition [11-16]. Apart from influencing matrix synthesis, several studies have shown that GlcN is also able to interfere with enzymatic matrix degradation [5,10,12,17-21]. These conflicting results can have various causes. It is debated whether glucosamine-sulphate (GlcN-S), glucosamine-hydrochloride (GlcN-HCl) and N-acetyl-glucosamine (GlcN-Ac) have similar effects on cartilage or not. Differences in the results can also be explained by the variety of culture models (e.g., monolayer vs pellet culture) and the variation in culture durations that have been used. Most studies were cultured for a relatively short period, so the chondrocytes were not yet surrounded by an extracellular matrix (ECM) and hence the effect of GlcN is tested in an anabolic state. To test the effect of GlcN in a catabolic situation ECM degrading substances, e.g., interleukin-1β (IL-1β), lipopolysaccharide or retinoic acid, are used by several investigators to mimic an OA like environment [5,7,11-13,15,16,18-25].

The aim of this series of experiments was to determine the effects of different GlcN derivatives on chondrocyte matrix production in a three-dimensional (3D) culture model: 1) Under mainly anabolic conditions where ECM still has to be formed, 2) In a situation of balance between GAG synthesis and degradation where ECM already has been formed, 3) Under catabolic conditions, through the addition of IL-1β. For all conditions we cultured chondrocytes in alginate beads since this is a widely accepted model to test chondrocyte behavior in a 3D environment [26].

Since some authors have postulated that competition between GlcN and glucose (Gluc) might be the basis for the mechanism of action of GlcN, we also tested the effect of increasing Gluc levels in the culture medium [9,18,27,28].

MATERIALS AND METHODS

Alginate cultures

Cartilage slices were dissected from the metacarpo-phalangeal joint of 6-12 months old calves under aseptically conditions and washed with physiological saline. Slices were incubated with pronase E in physiological saline (2 mg/ml; Sigma, St Louis, MO) for 1 h at 37°C followed by overnight incubation with collagenase B (1.5 mg/ml; Roche diagnostics Mannheim, Germany) in medium with 10% fetal calf serum (FCS). Undigested parts were removed using a 100 µm filter and isolated chondrocytes were washed with physiological saline. Cell viability was tested using the trypan blue exclusion test. Cells were suspended at a density of 4×10^6 cells/ml in sterile saline containing 1.2% low-viscosity alginate (Keltone LV, Kelco), and slowly, in a dropwise fashion, pressed through a 23-gage needle into a 102 mM $CaCl_2$ solution. After instantaneous gelation, the beads were allowed to polymerize further for a period of 10 min in the $CaCl_2$ solution. Then, beads were washed with saline and cultured with 10 beads per well in 24-well plates with 500 µl DMEM (Gibco, Grand Island, NY) low Gluc (1000mg/l, l = 5.55 mM) supplemented with 10% FCS, 50 µg/ml gentamicin, 1.5 µg/ml fungizone and 25 µg/ml freshly added L-ascorbic acid-2-phosphate. After a 3 day recovery period (pre-culture) the experiment was started. In all experiments medium was changed three times a week.

Experimental design

Experiment 1: GlcN effects on chondrocytes already surrounded by ECM

The purpose of this experiment was to test the effect of Gluc (Sigma, St Louis, MO), GlcN-HCl (Sigma, St Louis, MO), GlcN-S (Sigma, St Louis, MO) and GlcN-Ac (Sigma, St Louis, MO) on bovine chondrocytes in a situation of balance between GAG synthesis and degradation, i.e., in the presence of a preexisting ECM. Therefore experiment 1 started with a prolonged pre-culture period of 21 days (days 3-24) without any experimental interference. Then, treatment started by adding Gluc 5 mM or GlcN derivatives (0.1, 0.5 and 5 mM) to the culture medium for 14 days (days 24-38). This experiment was repeated three times (N = 3). For each experimental condition, three samples of three beads each were harvested at day 38 and biochemical analysis was performed as described later.

An earlier study showed that the typical chondrocytic appearance and spherical shape of bovine chondrocytes cultured in alginate beads are retained for at least 5 weeks [26]. We therefore decided that a 38 day culture period was within a safe margin for investigating real chondrocyte behavior.

Experiment 2a: GlcN effects in anabolic conditions

In order to test the effect of GlcN on bovine chondrocytes under mainly anabolic conditions, i.e., where the ECM still has to be formed, the alginate beads were treated for 14 days as in

experiment 1 but with treatment starting on day 3 (days 3-17). This experiment was performed three times (N = 3). Three samples of three beads per condition were harvested at day 17 for biochemical analysis.

Experiment 2b: GlcN effects in anabolic conditions: a time course

Here, the effect of GlcN on the developing ECM during the first 17 days of culture was studied in detail in time. Culture was performed similar to experiment 2a, except that beads were harvested at days 3, 6, 10 and 17. As a measure for ECM development, we investigated total amount of GAG per bead. This experiment was performed twice for the 5 mM conditions only (N = 2).

Experiment 3: GlcN effects in catabolic conditions

In this experiment IL-1β addition is used as a model to study the effects of GlcN under catabolic conditions. On day 8, the 10% FCS in the medium was washed out in three washing steps prior to culturing beads in low Gluc DMEM supplemented with 2% FCS with or without 10 ng/ml IL-1β for 48 h. On day 10 the IL-1β was washed out using the same washing protocol. From day 10 to 17 the alginate beads were again cultured in medium with 10% FCS. In this series of experiments four experimental conditions were created:

1) control culture for 17 days in low Gluc medium;
2) IL-1β treatment for 48 h, starting on day 8, as described above;
3) as in condition 1, but with 5 mM GlcN-HCl added on day 6 for 96 h;
4) as in condition 2, but with 5 mM GlcN-HCl added on day 6 for 96 h.

This experiment was repeated three times (N = 3). Three samples of three beads each were harvested for each condition at days 6, 8, 10, 13 and 17 for biochemical analysis.

Experiment 4: GlcN effects with increasing medium Gluc concentration

The purpose of experiment 4 was to investigate the alleged competition between GlcN and Gluc. This was tested in the situation without a preexisting ECM, since we expected that chondrocytes in this anabolic situation are highly in need for both Gluc and GlcN, so competition was expected to be the highest in this situation. As in experiment 2a, experimental treatment started immediately on day 3 for 14 days, with 5 mM GlcN-HCl and GlcN-Ac. An additional 5, 10 or 20 mM Gluc was added to the basal Gluc level of 5.55 mM in the culture medium, resulting in final concentrations of 5, 10, 15 and 25 mM Gluc. This experiment was repeated twice (N = 2). On day 17, three samples of three beads each were harvested and the amount of GAG measured.

In Fig. 1, the time course of addition of GlcN and IL-1β in the four experiments with respect to the ECM status, based on the data of the control conditions in experiment 1 and 2b, is displayed.

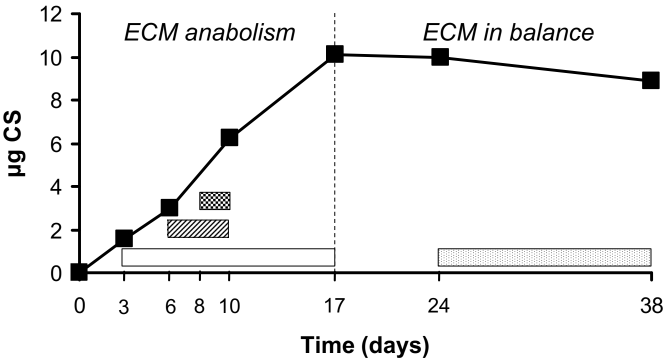

Figure 1. ECM status (based on the data of the control conditions from experiments 1 and 2b) and time course of experimental conditions. [dotted box] GlcN addition in experiment 1. [open box] GlcN addition in experiment 2 and 4. [hatched box] GlcN addition in experiment 3. [checkered box] IL-1β treatment in experiment 3.

Biochemical analysis of DNA and GAG amount

Beads were dissolved by adding 100 μl of 55 mM sodium citrate in 150 mM NaCI per bead for 15 min at 4°C. After addition of an equal volume of papain (Sigma, St Louis, MO), resulting in a concentration of 125 μg/ml, the beads were incubated overnight at 60°C. After the overnight incubation the samples were vortexed and incubated for another 2 hours at 60°C.

To evaluate the total number of chondrocytes present in the beads at the given time points, the amount of DNA was measured. The amount of DNA was quantified in a spectrofluorometer (Perkin-Elmer LS-2B) by its 365 nm/440 nm ratio using Hoechst 33258 dye with calf thymus DNA (Sigma, St Louis, MO) as a standard [29].

The results in our previous study indicated that addition of GlcN might lead to inhibition of matrix synthesis as well as degradation [30]. For this study we have chosen to quantify the total amount of GAG present in the beads using the DMB assay to provide us with the resultant of GAG production and degradation.

The amount of GAG was quantified using a modified Farndale assay in microtiter plates, with the pH of the dimethylmethylene blue solution set to 1.5 to prevent interference with alginate [31,32]. Briefly, the metachromatic reaction of GAG with dimethylmethylene blue is monitored by its A_{540}/A_{595} ratio in a spectrophotometer. Chondroitin sulphate (CS) C (Shark; Sigma, St Louis, MO) was used as a standard. If necessary, samples were diluted to be sure to measure within the linear range of the assay.

Data analysis

In the experiments, data from the individual experimental conditions consisted of the analysis of three samples of three beads each. The means of these data of each experiment were used to calculate and display mean and standard deviations for the amount of DNA and GAG (N=3 for experiment 1, 2a and 3; N=2 for experiment 2b and 4). For statistical analysis a Kruskal-Wallis test with post-hoc Mann-Whitney U test was performed using the data of all individual samples

using SPSS 11.0.1 (SPSS Inc., Chicago, IL). Thus statistics are based on nine samples (N=3 x 3 for experiment 1, 2a and 3) and six samples (N=2 x 3 for experiment 2b and 4) respectively. A p-value < 0.05 was considered to indicate statistically significant differences.

RESULTS

Experiment 1: GlcN effects on chondrocytes already surrounded by ECM

When GlcN is added for 14 days to chondrocytes that had first been allowed to form an ECM (addition from day 24 to 38 of culture) no statistically significant effect on the amount of GAG per bead was found compared to the control condition, except for 5 mM GlcN-Ac (Fig. 2A). Addition of GlcN-Ac showed a dose-dependent trend, with a 40 % increase in the amount of GAG per bead for the highest concentration, 5 mM GlcN-Ac ($p = 0.003$). Considering the amount of DNA per bead (Fig. 2B), only addition of 5 mM GlcN-S led to a small but statistically significant increase compared to the control condition (0.81 ± 0.03 µg vs 0.74 ± 0.07 µg; $p < 0.001$).

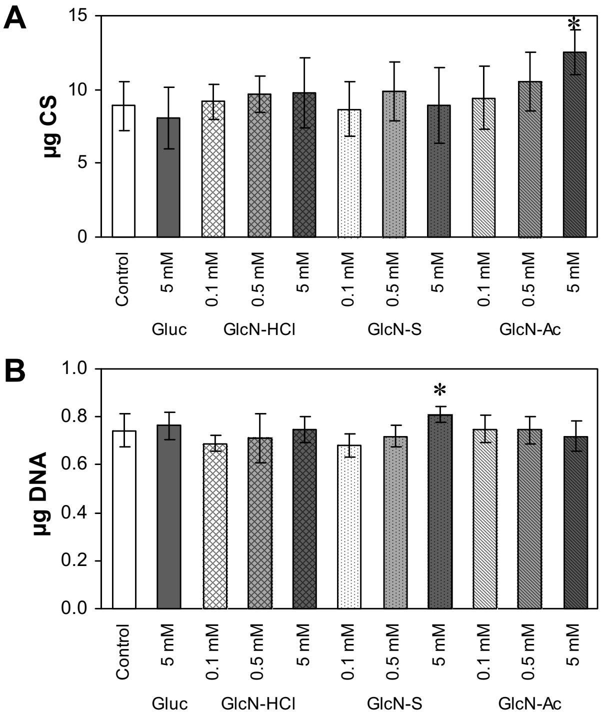

Figure 2. Total amount of GAG per bead (expressed as µg CS; panel A) and total amount of DNA per bead (panel B) on day 38 after stimulation with GlcN. Three concentrations of three different forms of GlcN were added during the last 14 days of culture. An ECM was preformed during the first 24 days and the effects of GlcN are thus tested in a situation where a balance between synthesis and degradation exists. * indicates a statistically significant difference ($p < 0.05$), compared to the nonsupplemented control.

Experiment 2a: GlcN effects in anabolic conditions

The amount of GAG per bead was significantly reduced with 0.5 mM GlcN-HCl (9.46 ± 1.7 µg vs 10.07 ± 2.3 µg; p = 0.04), 5 mM GlcN-HCl (4.44 ± 1.78 µg; p = 0.001), 0.5 mM GlcN-S (6.55 ± 0.18 µg, p = 0.024) and 5 mM GlcN-S (2.76 ± 0.32 µg, p < 0.001) compared to the control. The effect of the different GlcN derivatives on the GAG content per bead is displayed in Fig. 3A. The result of the different GlcN derivatives on the amount of DNA per bead is shown in Fig. 3B. A dose-dependent relation was found for GlcN-HCl and GlcN-S, i.e., with increasing GlcN concentration, the total amount of DNA per bead decreases. For 5 mM GlcN-HCl and GlcN-S this decrease in DNA content per bead was statistically significant.

Addition of 5 mM Gluc to chondrocytes *in vitro* from day 3 to 17 did not significantly influence the GAG or DNA content per bead, nor did any of the tested concentrations of GlcN-Ac.

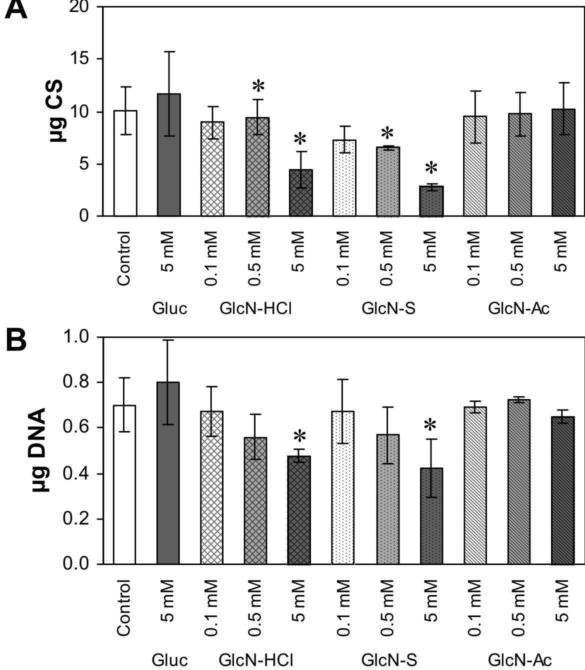

Figure 3. Total GAG content (panel A) and total DNA content (panel B) per bead upon GlcN treatment on day 17. Chondrocytes were stimulated in the absence of an ECM and the GlcN effects are thus tested in an anabolic situation. Three different forms of GlcN, three concentrations each, were added during the last 14 days of culture. * indicates a statistically significant difference (p < 0.05), compared to the nonsupplemented control.

Experiment 2b: GlcN effects in anabolic conditions: a time course

The decreased amount of DNA and GAG per bead upon addition of GlcN-S and GlcN-HCl during the anabolic phase of culture as observed in experiment 2a was further evaluated in a time course study. For this experiment we used a limited number of conditions (5 mM GlcN-HCl, 5

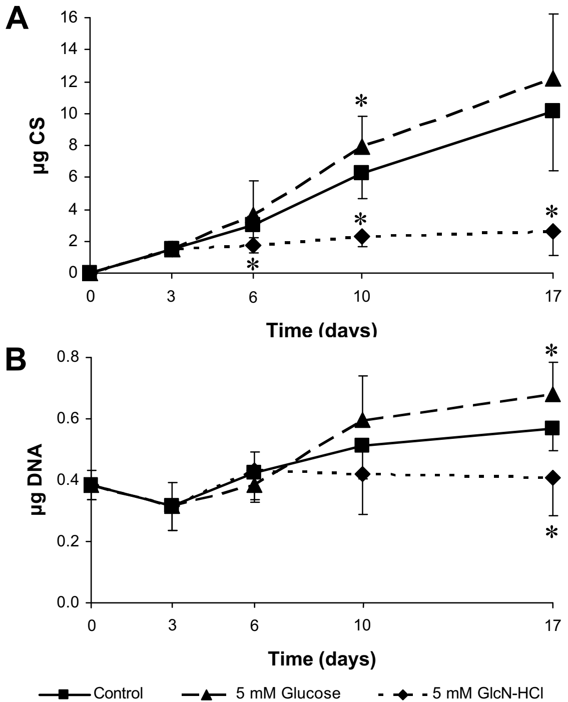

Figure 4. Changes in GAG per bead (panel A) and DNA per bead (panel B) upon GlcN treatment during 17 days of culture. Cultures were treated with 5 mM Gluc and 5 mM GlcN-HCl during the last 14 days. Beads were harvested on day 3, 6, 10, 17. In the absence of ECM, the GlcN effects are tested in an anabolic situation. * indicates a statistically significant difference ($p < 0.05$), compared to the nonsupplemented control.

mM Gluc, and control), but the culture scheme was similar to experiment 2a. The amount of GAG and DNA per bead increases in time in the control condition (Fig. 4). Addition of GlcN-HCl from the start of the experiment inhibited this increase. The reduction in the amount of GAG per bead compared to the control was already statistically significant at day 6 (1.73 ± 0.46 µg vs 3.01 ± 0.76 µg; $p < 0.001$). Addition of 5 mM Gluc led to an even higher GAG content per bead when compared to the control, but only statistically significant at day 10 (7.92 ± 1.92 µg vs 6.23 ± 1.57 µg; $p = 0.008$). An effect on the amount of DNA per bead became only significant at day 17. GlcN-HCl reduced (0.41 ± 0.12 µg vs 0.57 ± 0.07 µg; $p = 0.004$) and 5 mM Gluc increased the amount of DNA per bead when compared to the control condition (0.68 ± 0.10 µg vs 0.57 ± 0.07 µg; $p = 0.016$).

Experiment 3: GlcN effects in catabolic conditions

Addition of 10 ng/ml IL-1β from day 8 till day 10 decreases the total amount of GAG per bead, reaching the maximal effect between day 10 and day 13 (Fig. 5). On day 13, the total amount of GAG per bead significantly differs from the control (7.59 ± 1.28 µg vs 10.49 ± 1.33 µg; $p <0.001$).

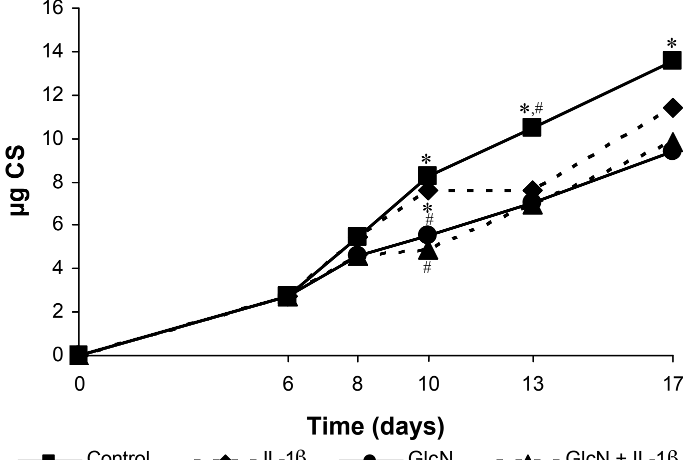

Figure 5. GlcN pre-treatment protects against IL-1β triggered changes in total GAG per bead. GAG content per bead during 17 days of culture is displayed for the control condition (Control), after stimulation on day 8 for 48 h with 10 ng/ml IL-1β (IL-1β), after treatment with 5 mM GlcN-HCl on day 6 for 96 h (GlcN), and after treatment with 5 mM GlcN-HCl on day 6 for 96 h, followed by stimulation with 10 ng/ml IL-1β on day 8 for 48 h (GlcN + IL-1β). Beads were harvested on day 6, 8, 10, 13, 17. * indicates a statistically significant difference ($p < 0.05$), compared to 5 mM GlcN-HCl treatment (GlcN). # indicates a statistically significant difference ($p < 0.05$), compared to 10 ng/ml IL-1β (IL-1β).

Between day 13 and day 17 the amount of GAG per bead in the IL-1β treatment group recovers by increasing at a faster rate than the control group.

As expected, based on our earlier results, the addition of 5 mM GlcN-HCl on day 6 for 96 h led to a reduced total amount of GAG per bead when compared to the nonsupplemented control, reaching statistical significance from day 10 onwards. Addition of 10 ng/ml IL-1β to the alginate beads pre-treated with 5 mM GlcN-HCl, did not lead to a statistically significant further reduction in the total amount of GAG per bead (Fig. 5). Thus, pre-treatment with 5 mM GlcN-HCl seems to protect chondrocytes against the IL-1β triggered, destructive effects observed without GlcN treatment.

Experiment 4: GlcN effects with increasing medium Gluc concentration

Here, we investigated the alleged competition between Gluc and GlcN. Overall, the GAG content per bead increased with increasing concentrations of extracellular Gluc, (Fig. 6). The GlcN-HCl condition revealed an increase in GAG per bead that dose-dependently correlates with the Gluc concentration. This Gluc dependent increase in GAG content was far less pronounced in the control and the GlcN-Ac condition (Fig. 6). In the presence of 15 or 25 mM Gluc, GlcN-HCl no longer significantly reduced the GAG content per bead compared to the control condition at 5 mM Gluc.

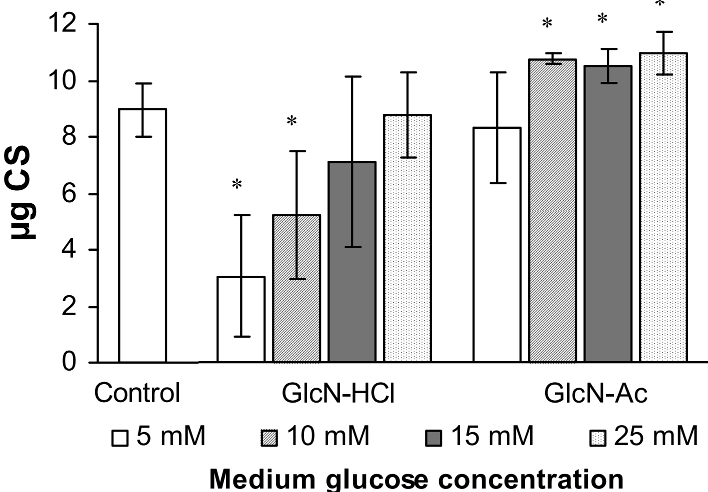

Figure 6. Influence of medium Gluc concentration on GlcN triggered changes in GAG content per bead. In this experimental set-up, the alleged competition between GlcN and Gluc was tested on day 17 of culture; different cultures with either GlcN-HCl or GlcN-Ac were simultaneously treated with increasing concentrations of Gluc (5-25 mM). * indicates a statistically significant difference ($p < 0.05$), compared to control condition at 5 Mm medium Gluc concentration.

DISCUSSION

Addition of GlcN-HCl or GlcN-S did not have a significant effect on total GAG content per bead when added *in vitro* to ECM surrounded chondrocytes (experiment 1). In an anabolic culture environment, addition of 5 mM GlcN-HCl and GlcN-S even reduced the GAG content per bead compared to the control condition. In contrast, GlcN-Ac did not show this effect. It even appeared to have a small stimulatory effect on GAG content in ECM surrounded chondrocytes (experiments 1 and 2). The apparently inhibitive effect of GlcN-HCl and GlcN-S on GAG content could be diminished by increasing the extracellular Gluc concentration (experiment 4), indicating an interference with Gluc metabolism. On the other hand, this interference also seems to prevent the catabolic effects induced by 10 ng/ml IL-1β (experiment 3).

In an earlier study with GlcN added to human OA explant cultures, we found down-regulation of marker genes for anabolic (collagen II and aggrecan) and catabolic (a disintegrin and metalloproteinase with a thrombospondin type motif (ADAMTSs), and matrix metalloproteinases (MMPs)) processes [30]. For the MMPs we showed this by measuring protein activity, but the effect of GlcN derivatives on GAG content was not investigated in this earlier study. In accordance with our previous study we have now shown that addition of both GlcN-S and GlcN-HCl also leads to less GAG production, when added to isolated chondrocytes *in vitro*. Furthermore we showed that treatment of chondrocytes with GlcN leads to protection against catabolic processes that were induced by adding IL-1β. This, again, is in agreement with our previous findings on osteoarthritic explants. In a recent study by Gouze et al., where addition

of 20 mM GlcN-HCl to rat chondrocytes led to protection against IL-1β induced expression of genes known to be involved in matrix degradation these results were again confirmed [33]. GlcN-HCl did not seem to stimulate the expression of genes involved in matrix synthesis. In an earlier article, Gouze et al. investigated the effect of different concentrations of GlcN varying between 4.6 mM and 20.9 mM and IL-1β on proteoglycan synthesis, using rat chondrocytes in alginate, in the presence of 5.5 mM Gluc, the same medium Gluc concentration we used in our experiments [19]. They found that, except for the lowest 4.6 mM concentration, GlcN treatment significantly reduced the inhibition of proteoglycan synthesis by IL-1β. These results are fairly consistent with our data from experiment 3. The main difference between our results and those in the two mentioned studies by Gouze et al. is that we have shown significant effects with a 5 mM GlcN concentration, which is far lower than the supraphysiological dose used by Gouze and co-workers.

We found that total amount of GAG was lower when 5 mM GlcN-HCl or GlcN-S was added under anabolic culture conditions to the alginate embedded chondrocytes, and that GlcN-Ac did not show such an effect (experiment 2). For the amount of DNA, similar results were found. These results are a general confirmation of what Terry et al. found in their study [34]. They found a dose-dependent inhibition of cell proliferation and proteoglycan synthesis upon addition of 0.1-5 mM GlcN-HCl in their anchorage-dependent cultures. Addition of GlcN-Ac in this culture system did not lead to inhibition. For the anchorage-independent culture system, i.e., alginate beads, the inhibitory effects of GlcN-HCl on cell proliferation were also present, but not as prominent. These findings of Terry et al. are similar to what we have shown in figure 4.

The decrease in cell proliferation is also in accordance with the recent results by Varghese et al., who found a decrease in cell proliferation with increasing GlcN-HCl concentration using a 3D cell culture system [35]. Using the same system they found that GAG production was maximized at 2 mM and was inhibited at higher concentrations. In contrast with these results, we found, using a different 3D culture system, that total amount of GAG was already significantly lower compared to the control condition when 0.5 mM GlcN-HCl was added and that this difference only increased when GlcN-HCl concentration was 5 mM (Fig. 3).

As in a lot of other *in vitro* studies, the major concern in the interpretation of our data with respect to clinical applicability is the concentration of GlcN used in our experimental set-up. A recent study on oral bioavailability and plasma pharmokinetics of GlcN in man with the standard 1500 mg once-daily dosage, showed a peak plasma level of about 10 μM [36]. The GlcN concentrations we used in our experiments were much higher. The reason we decided to use GlcN concentrations up to 5 mM was mainly based on the findings of Mroz and Silbert combined with the medium Gluc concentration we used [9,27]. Mroz and Silbert suggested that not only the absolute concentration of GlcN, but the GlcN to Gluc ratio plays an important role in the utilization and therefore efficacy of exogenously provided GlcN. In two studies using mouse chondrocytes or immortalized human chondrocytes *in vitro*, exogenous GlcN was incorporated in newly formed CS when it was added at equimolar concentrations with Gluc in the

culture medium. Chondrocytes preferably incorporated GlcN that was endogenously formed from Gluc into CS when medium Gluc concentration became higher than the exogenous GlcN concentration. Since mean Gluc concentration in osteoarthritic knee-joint effusions was shown to be 5.4 mM, we used medium with low Gluc concentration (5.55 mM) in order to mimic the physiological situation [37]. Therefore, we decided to add GlcN to the medium at concentrations equimolar to, or lower than that of medium Gluc.

It still remains highly questionable which levels of GlcN can be reached in articular cartilage and how high concentrations need to be to exert effects *in vivo*. In another study on bioavailability, administration of GlcN-HCl at clinically relevant levels to horses in a single dose led to a synovial fluid concentration of less than 10% of that measured in serum at the same time [38]. However, after 6 h when GlcN was almost completely cleared from serum, it could still be detected in the synovial fluid. Earlier studies with radio labeled GlcN showed a special capacity of articular cartilage to accumulate and retain GlcN in animals and man [39-42]. Due to this capacity, GlcN concentration surrounding the chondrocyte might be much higher than that measured in synovial fluid or serum. As we mentioned above, the importance of the GlcN to Gluc ratio on the effectiveness of exogenous GlcN was already stressed by other authors. With this ratio in mind and the fact that Windhaber et al. measured a Gluc concentration directly surrounding the chondrocytes of 1 mM, peri-cellular GlcN concentration probably does not even have to reach 5 mM to be effective *in vivo* [28].

The mechanism of action of GlcN might be explained by interference of GlcN with Gluc utilization. GlcN-HCl and GlcN-S seem to inhibit GAG synthesis, but adding increasing concentrations of Gluc can reverse this effect (experiment 4). In contrast, GlcN-Ac does not have such inhibitory effects. A possible explanation for this might be that Gluc and GlcN-HCl and GlcN-S can all be transported into cells by members of the GLUT family, a Gluc transporter system [43]. Therefore, GlcN can act as a competitive inhibitor for Gluc uptake [28]. Also, intracellular competition may occur at substrate level, since Gluc and GlcN are both phosphorylated by the same enzyme: glucokinase [44]. Phosphorylated GlcN in turn is an allosteric inhibitor of glucokinase, which results in a negative feedback loop [45]. Due to the direct competition between Gluc and GlcN-HCl or GlcN-S at these different cellular levels, less Gluc-6-P may become available to enter the glycolysis. It is commonly known that for optimal adenosine triphosphate (ATP) production, a regular Gluc supply is needed since chondrocytes are highly glycolytic cells [46]. So, by (partially) blocking the glycolysis with exogenous GlcN-HCl or GlcN-S, the intracellular ATP levels will eventually drop. This effect of exogenous GlcN on intracellular ATP levels has been described earlier [47,48]. And in addition, other studies have showed that depleting intracellular ATP, leads to a decrease of proteoglycan synthesis [49,50]. Thus with al these earlier studies in mind, we hypothesize that GlcN-HCl or GlcN-S addition might lead to intracellular ATP depletion, which results in less energy for all intracellular processes like, among others, ECM production and breakdown. The explanation why GlcN-Ac does not show an inhibitory effect on matrix

synthesis may be that it is phosphorylated by a different enzyme and that no competition with Gluc occurs, while its phosphorylated product can still be used for GAG synthesis [51].

Addition of more Gluc might reverse the competitive GlcN effects, leading to a restored energy supply eventually normalizing GAG synthesis, as could be observed in experiment 4. The latter is supported by data from Sandy et al. who found that the degree of inhibition of aggrecanase response in rat chondrocytes by GlcN was much reduced when medium Gluc was increased from 5 mM to 15-25 mM [18].

In summary, we conclude that addition of GlcN may interfere with Gluc utilization and thereby compromising both anabolic and catabolic processes. In the current study, this was demonstrated for anabolic processes by a decreased ECM synthesis and for catabolic processes by a protection against IL-1β mediated ECM breakdown. Based on these results, we suggest that the claimed structure modifying effects of GlcN are more likely based on protection against ECM degradation than production of new ECM.

ACKNOWLEDGEMENT

We would like to thank Rodi Horstman for his excellent work with the IL-1β experiments.

REFERENCES

1. Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, *et al.* Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. N Engl J Med 2006;354:795-808.
2. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, *et al.* Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-6.
3. Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002;162:2113-23.
4. Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-34.
5. Dodge GR, Jimenez SA. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis Cartilage 2003;11:424-32.
6. Bassleer C, Henrotin Y, Franchimont P. In-vitro evaluation of drugs proposed as chondroprotective agents. Int J Tissue React 1992;14:231-41.
7. Lippiello L. Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress. Osteoarthritis Cartilage 2003;11:335-42.
8. Vidal y Plana RR, Bizzarri D, Rovati AL. Articular cartilage pharmacology: I. In vitro studies on glucosamine and non steroidal antiinflammatory drugs. Pharmacol Res Commun 1978;10:557-69.
9. Mroz PJ, Silbert JE. Use of (3)H-glucosamine and (35)S-sulfate with cultured human chondrocytes to determine the effect of glucosamine concentration on formation of chondroitin sulfate. Arthritis Rheum 2004;50:3574-9.
10. Fenton JI, Chlebek-Brown KA, Caron JP, Orth MW. Effect of glucosamine on interleukin-1-conditioned articular cartilage. Equine Vet J Suppl 2002:219-23.
11. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:444-51.
12. Orth MW, Peters TL, Hawkins JN. Inhibition of articular cartilage degradation by glucosamine-HCl and chondroitin sulphate. Equine Vet J Suppl 2002:224-9.
13. de Mattei M, Pellati A, Pasello M, de Terlizzi F, Massari L, Gemmati D, *et al.* High doses of glucosamine-HCl have detrimental effects on bovine articular cartilage explants cultured in vitro. Osteoarthritis Cartilage 2002;10:816-25.
14. Anderson CC, Cook JL, Kreeger JM, Tomlinson JL, Wagner-Mann CC. In vitro effects of glucosamine and acetylsalicylate on canine chondrocytes in three-dimensional culture. Am J Vet Res 1999;60: 1546-51.
15. Ilic MZ, Martinac B, Handley CJ. Effects of long-term exposure to glucosamine and mannosamine on aggrecan degradation in articular cartilage. Osteoarthritis Cartilage 2003;11:613-22.
16. Gouze JN, Bianchi A, Becuwe P, Dauca M, Netter P, Magdalou J, *et al.* Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-kappa B pathway. FEBS Lett 2002;510:166-70.
17. Piperno M, Reboul P, Hellio Le Graverand MP, Peschard MJ, Annefeld M, Richard M, *et al.* Glucosamine sulfate modulates dysregulated activities of human osteoarthritic chondrocytes in vitro. Osteoarthritis Cartilage 2000;8:207-12.
18. Sandy JD, Gamett D, Thompson V, Verscharen C. Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. Biochem J 1998;335 (Pt 1):59-66.
19. Gouze JN, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, *et al.* Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis:

influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. Arthritis Rheum 2001;44:351-60.
20. Byron CR, Orth MW, Venta PJ, Lloyd JW, Caron JP. Influence of glucosamine on matrix metalloproteinase expression and activity in lipopolysaccharide-stimulated equine chondrocytes. Am J Vet Res 2003;64:666-71.
21. Mello DM, Nielsen BD, Peters TL, Caron JP, Orth MW. Comparison of inhibitory effects of glucosamine and mannosamine on bovine articular cartilage degradation in vitro. Am J Vet Res 2004;65:1440-5.
22. Shikhman AR, Kuhn K, Alaaeddine N, Lotz M. N-acetylglucosamine prevents IL-1 beta-mediated activation of human chondrocytes. J Immunol 2001;166:5155-60.
23. Nakamura H, Shibakawa A, Tanaka M, Kato T, Nishioka K. Effects of glucosamine hydrochloride on the production of prostaglandin E2, nitric oxide and metalloproteases by chondrocytes and synoviocytes in osteoarthritis. Clin Exp Rheumatol 2004;22:293-9.
24. Largo R, Alvarez-Soria MA, Diez-Ortego I, Calvo E, Sanchez-Pernaute O, Egido J, *et al.* Glucosamine inhibits IL-1beta-induced NFkappaB activation in human osteoarthritic chondrocytes. Osteoarthritis Cartilage 2003;11:290-8.
25. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:258-65.
26. Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar EJ. Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. Matrix 1992;12:116-29.
27. Mroz PJ, Silbert JE. Effects of [3H]glucosamine concentration on [3H]chondroitin sulphate formation by cultured chondrocytes. Biochem J 2003;376:511-5.
28. Windhaber RA, Wilkins RJ, Meredith D. Functional characterisation of glucose transport in bovine articular chondrocytes. Pflugers Arch 2003;446:572-7.
29. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 1988;174:168-76.
30. Uitterlinden EJ, Jahr H, Koevoet JL, Jenniskens YM, Bierma-Zeinstra SM, Degroot J, *et al.* Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. Osteoarthritis Cartilage 2006;14:250-7.
31. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173-7.
32. Enobakhare BO, Bader DL, Lee DA. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. Anal Biochem 1996;243:189-91.
33. Gouze JN, Gouze E, Popp MP, Bush ML, Dacanay EA, Kay JD, *et al.* Exogenous glucosamine effectively protects chondrocytes from the arthritogenic effects of IL-1beta. Arthritis Res Ther 2006;8:R173.
34. Terry DE, Rees-Milton K, Smith P, Carran J, Pezeshki P, Woods C, *et al.* N-acylation of glucosamine modulates chondrocyte growth, proteoglycan synthesis, and gene expression. J Rheumatol 2005;32:1775-86.
35. Varghese S, Theprungsirikul P, Sahani S, Hwang N, Yarema KJ, Elisseeff JH. Glucosamine modulates chondrocyte proliferation, matrix synthesis, and gene expression. Osteoarthritis Cartilage 2006.
36. Persiani S, Roda E, Rovati LC, Locatelli M, Giacovelli G, Roda A. Glucosamine oral bioavailability and plasma pharmacokinetics after increasing doses of crystalline glucosamine sulfate in man. Osteoarthritis Cartilage 2005;13:1041-9.
37. Krachler M, Domej W. Clinical laboratory parameters in osteoarthritic knee-joint effusions correlated to trace element concentrations. Biol Trace Elem Res 2001;79:139-48.
38. Laverty S, Sandy JD, Celeste C, Vachon P, Marier JF, Plaas AH. Synovial fluid levels and serum pharmacokinetics in a large animal model following treatment with oral glucosamine at clinically relevant doses. Arthritis Rheum 2005;52:181-91.
39. Setnikar I, Giacchetti C, Zanolo G. Pharmacokinetics of glucosamine in the dog and in man. Arzneimittelforschung 1986;36:729-35.

40. Setnikar I, Giachetti C, Zanolo G. Absorption, distribution and excretion of radioactivity after a single intravenous or oral administration of [14C] glucosamine to the rat. Pharmatherapeutica 1984;3:538-50.
41. Setnikar I, Rovati LC. Absorption, distribution, metabolism and excretion of glucosamine sulfate. A review. Arzneimittelforschung 2001;51:699-725.
42. Setnikar I, Palumbo R, Canali S, Zanolo G. Pharmacokinetics of glucosamine in man. Arzneimittelforschung 1993;43:1109-13.
43. Uldry M, Ibberson M, Hosokawa M, Thorens B. GLUT2 is a high affinity glucosamine transporter. FEBS Lett 2002;524:199-203.
44. Van Schaftigen E. Glucosamine-sensitive and -insensitive detritiation of [2-3H]glucose in isolated rat hepatocytes: a study of the contributions of glucokinase and glucose-6-phosphatase. Biochem J 1995;308 (Pt 1):23-9.
45. Virkamaki A, Yki-Jarvinen H. Allosteric regulation of glycogen synthase and hexokinase by glucosamine-6-phosphate during glucosamine-induced insulin resistance in skeletal muscle and heart. Diabetes 1999;48:1101-7.
46. Otte P. Basic cell metabolism of articular cartilage. Manometric studies. Z Rheumatol 1991;50:304-12.
47. Tesoriere G, Vento R, Calvaruso G. Inhibitory effect of D-glucosamine on glycolysis in bovine retina. Biochim Biophys Acta 1975;385:58-67.
48. Hresko RC, Heimberg H, Chi MM, Mueckler M. Glucosamine-induced insulin resistance in 3T3-L1 adipocytes is caused by depletion of intracellular ATP. J Biol Chem 1998;273:20658-68.
49. Lee RB, Urban JP. Evidence for a negative Pasteur effect in articular cartilage. Biochem J 1997;321 (Pt 1):95-102.
50. Baker MS, Feigan J, Lowther DA. The mechanism of chondrocyte hydrogen peroxide damage. Depletion of intracellular ATP due to suppression of glycolysis caused by oxidation of glyceraldehyde-3-phosphate dehydrogenase. J Rheumatol 1989;16:7-14.
51. Hinderlich S, Berger M, Schwarzkopf M, Effertz K, Reutter W. Molecular cloning and characterization of murine and human N-acetylglucosamine kinase. Eur J Biochem 2000;267:3301-8.

Chapter 4

Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants

E.J. Uitterlinden, J.L.M. Koevoet, C.F. Verkoelen †, S.M.A. Bierma-Zeinstra, H. Jahr, H. Weinans, J.A.N. Verhaar, G.J.V.M. van Osch

BMC Musculoskelet Disord 2008 Sep 11; 9:120

ABSTRACT

Background

Glucosamine (GlcN) used by patients with osteoarthritis was demonstrated to reduce pain, but the working mechanism is still not clear. Viscosupplementation with hyaluronic acid (HA) is also described to reduce pain in osteoarthritis. The synthesis of HA requires GlcN as one of its main building blocks. We therefore hypothesized that addition of GlcN might increase HA production by synovium tissue.

Methods

Human osteoarthritic synovium explants were obtained at total knee surgery and pre-cultured for 1 day. The experimental conditions consisted of a 2 days continuation of the culture with addition of N-acetyl-glucosamine (GlcN-Ac; 5 mM), glucosamine-hydrochloride (GlcN-HCl; 0.5 and 5 mM), glucose (Gluc; 0.5 and 5 mM). Hereafter HA production was measured in culture medium supernatant using an enzyme-linked binding protein assay. Real time RT-PCR was performed for hyaluronic acid synthase (*HAS*) 1, 2 and 3 on RNA isolated from the explants.

Results

0.5 mM and 5 mM GlcN-HCl significantly increased HA production compared to control (approximately 2 – 4-fold), whereas GlcN-Ac had no significant effect. Addition of 5 mM Gluc also increased HA production (approximately 2-fold), but 0.5 mM Gluc did not. Gene expression of the HA forming enzymes *HAS* 1, 2 and 3 was not altered by the addition of GlcN or Gluc.

Conclusions

Our data suggest that exogenous GlcN can increase HA production by synovium tissue and is more effective at lower concentrations than Gluc. This might indicate that GlcN exerts its potential analgesic properties through stimulation of synovial HA production.

INTRODUCTION

Glucosamine (GlcN) is popular among patients suffering from osteoarthritis (OA). Although the efficacy of GlcN in OA is still under debate, subgroup analysis in patients with moderate-to-severe pain in a recent large NIH trial indicated that glucosamine hydrochloride (GlcN-HCl) might have a positive effect on pain [1]. Two clinical studies indicated that GlcN might have structure modifying effects on cartilage in knee OA [2,3]. The claim of structure modifying properties of GlcN is mostly based on the effects on cartilage. However, cartilage is not innervated and thus pain reduction after GlcN administration, as has been reported in the previously mentioned NIH trial, can therefore not be explained by the effect of GlcN on cartilage tissue. Considering pain reduction in OA, viscosupplementation with hyaluronic acid (HA) has been shown to have beneficial effects on both pain and function in patients with knee OA [4]. Since GlcN is an important building block of HA and HA is found in high amounts in articular joints, increasing HA production through the administration of GlcN might be a way to explain the pain relieving effect of GlcN. HA is synthesized by hyaluronic acid synthases (*HAS*) which covalently link the monomeric building blocks glucuronic acid and N-acetyl-glucosamine (GlcN-Ac) in an alternating fashion. Hence, if the *HAS* enzymes are at hand, availability of its building blocks is another prerequisite for the synthesis of HA. But, more availability of substrate does not necessarily have to lead to more HA.

The aim of our study was to evaluate whether GlcN derivatives have an effect on HA production in a human synovium explant model. Specifically, we investigated if HA production can be stimulated by adding GlcN and if GlcN affects gene expression of the *HAS* enzymes.

METHODS

Explant culture

Human synovium was obtained from patients suffering from knee OA at time of total knee arthroplasty operation (12 patients, age 48-70 male:female ratio 1:2, with approval of the Ethical Committee number MEC 2004-140). To remove any remaining synovial fluid or blood, raw material was rinsed three times in physiological saline. From this raw material explants of approximately 3 mm^2 each were created with a scalpel and pooled in a Petri dish. Per condition, explants were randomly taken from the Petri dish and cultured in a 6-well plate with 3 ml culture medium per well. During the whole experiment this culture medium consisted of low glucose (1000mg/l l = 5.55 mM) D-MEM (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (containing 0.5 mM glucose), 50 µg/ml gentamicin, 1.5 µg/ml fungizone. The total amount of explants per patient differed. Therefore, care was taken to use an equal number of explants per culture condition within one patient. In the final analysis, results were corrected for total sample weight.

After a 24 hour pre-culture period (day 1), the culture medium was refreshed and the actual experiment was started by supplementing the culture medium with different derivatives for 48 hours (day 2-3).

In the first series of experiments (N=6 patients) the effect of different GlcN derivatives on HA production was studied. Therefore, the culture medium was supplemented with GlcN-HCl (Sigma, St. Louis, MO) or GlcN-Ac (Sigma, St. Louis, MO) at 5 mM.

In the second series of experiments (N=6 patients) we investigated the effect of different GlcN concentrations on HA production. We also investigated the effect of glucose (Gluc) concentrations similar to that of GlcN on HA production, since *in vivo* GlcN is being formed from Gluc. Finally, in the second series of experiments the effect of GlcN and Gluc on *HAS* gene expression was studied. In this second series of experiments the culture medium was supplemented with GlcN-HCl or Gluc (Sigma, St. Louis, MO) at 0.5 mM and 5 mM, respectively. In all experiments the culture medium without GlcN or extra Gluc supplementation was used as a control.

For all experiments, at day 3 medium supernatant was stored at −20°C for analysis, explants were harvested and the wet weight per sample was determined. The explants from experiment 2 were snap frozen in liquid nitrogen for RNA isolation.

Analysis

HA synthesis was measured in culture medium using an enzyme-linked binding protein assay (Hyaluronic Acid test Kit, Corgenix Inc., Westminster, CO) in a 96 wells plate. The wells were coated with HA binding protein from bovine cartilage to capture HA and an enzyme-conjugated version of HA binding protein to detect and measure HA in the sample using a spectrophotometer at 450 nm (650 nm reference). HA from rooster comb was used as standard. The resulting amount of HA in the culture medium was corrected for sample weight. For display purposes, these values were expressed relative to the control condition on day 3.

The wet weight per sample of the snap frozen explants from experiment 2 was determined and the frozen synovium was then processed using the Mikro-Dismembrator S® (B. Braun Biotech International GmbH, Melsungen, Germany). RNA was extracted using RNA-Bee™ (TEL-TEST, Inc; Friendswood, TX, USA) according to manufacturer's guidelines and subsequently precipitated with 2-propanol. RNA was further purified using RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) with on-column DNA-digestion. Total RNA was quantified accurately using a NanoDrop™ 1000 spectrophotometer (Nanodrop technologies, Wilmington, DE) according to manufacturer's instructions and 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). TaqMan® assays were performed on an ABI Prism 7000 for *HAS* 1, 2 and 3 using an assay-on-demand (Applied Biosystems, Foster City, CA, order numbers *HAS1*: Hs00758053_m1, *HAS2*: Hs00193435_m1, *HAS3*: Hs00193436_m1). Tissue processing and gene expression analysis were performed using the same protocol we described earlier in our study on human osteoarthritic cartilage explants [5].

For each patient the Ct-values of each control and experimental condition were normalized to glyceraldehyde 3-phosphate dehydrogenase. Hereafter each experimental condition was expressed relative to the corresponding control condition of the same patient, according to the $2^{-\Delta\Delta Ct}$ method described by Livak et al. [6,7]. The resulting number of this calculation indicated whether there was an up or down-regulation of gene expression of an experimental condition compared to its paired untreated control. Thereafter, for each subset of experimental conditions the median of these numbers was calculated. For graphical display purposes only the $2^{-\Delta\Delta Ct}$ values were expressed as a ^{10}LOG. For statistical analysis a Friedman test with post-hoc Wilcoxon signed ranks test was performed on the amount of HA in the culture medium corrected for sample weight (HA production) and on the normalized Ct values (gene expression), using SPSS 11.5.0 (SPSS Inc., Chicago, IL). A p-value ≤ 0.05 was considered to indicate statistically significant differences.

RESULTS

In the first set of experiments, the amount of HA in the medium was increased significantly 3.66 fold by the addition of 5 mM GlcN-HCl when compared to the control condition (Fig. 1). The increase after addition of 5 mM GlcN-Ac was not statistically significant.

In the second set of experiments, in which samples from 6 other patients were used, HA in the culture medium was significantly increased by 0.5 mM GlcN-HCl (204 %) and 5 mM GlcN-HCl (207 %) and by addition of 5 mM Gluc (178%), but not by 0.5 mM Gluc (Fig 2).

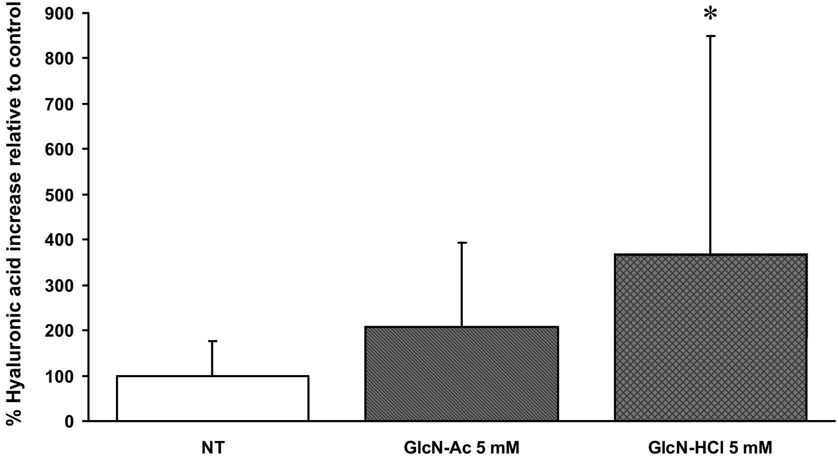

Figure 1. HA after addition of 5 mM GlcN-Ac or GlcN-HCl to human synovium explants. Amount of hyaluronic acid secreted into culture medium after 48 hours treatment of human osteoarthritic synovium explants with 5 mM GlcN-Ac (N=6) or 5 mM GlcN-HCl (N=6) for 2 days. Values are expressed relative to the non-treated (NT) control condition which is set at 100% (N=6, first bar). * indicates a statistically significant difference (p = 0.028).

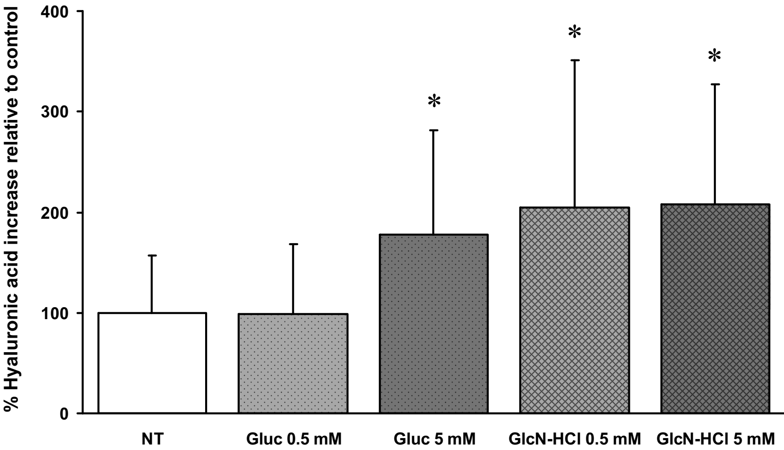

Figure 2. HA after addition of 0.5 and 5 mM GlcN-HCl or Gluc to human synovium explants. Amount of hyaluronic acid secreted into culture medium after 48 hours treatment of human osteoarthritic synovium explants with 0.5 mM GlcN-HCl (N=6), 5 mM GlcN-HCl (N=6), 0.5 mM Gluc (N=5) or 5 mM Gluc (N=6) for 2 days. Values are expressed relative to the non-treated (NT) control condition which is set at 100% (N=6, first bar). * indicates a statistically significant difference (p = 0.028).

Furthermore, all three *HAS* isoforms were expressed in the synovium explants. No statistically significant difference in gene expression was found for the experimental conditions, compared to the control condition (Fig. 3).

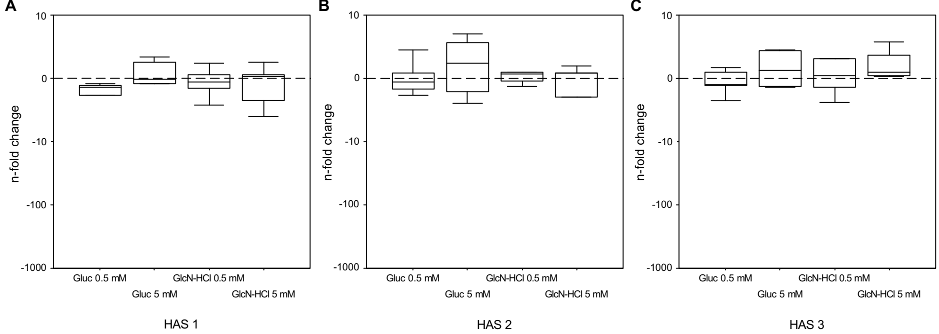

Figure 3. *HAS* gene expression after addition of GlcN-HCl or Gluc to human synovium explants. Change in *HAS* gene expression in human osteoarthritic synovium explants after culture with glucosamine or glucose. Synovium explants were pre-cultured for 1 day, followed by 2 days of treatment with 0.5 mM GlcN-HCl (N=6), 5 mM GlcN-HCl (N=5), 0.5 mM Gluc (N=5) and 5 mM Gluc(N=6). The n-fold change for *HAS1* (panel A), *HAS2* (panel B) and *HAS3* (panel C), normalized to glyceraldehyde 3-phosphate dehydrogenase and relative to the untreated control (indicated by the dotted line) is displayed on the vertical axis. Negative values indicate down-regulation and positive values indicate up-regulation of gene expression. No statistically significant differences were found.

DISCUSSION

Addition of 0.5 mM or 5 mM GlcN-HCl to human osteoarthritic synovium explants *in vitro* led to more HA in the medium when compared to the control condition. 5 mM GlcN-Ac did not have a significant effect on medium HA concentration.

To this date several *in vitro* studies have been published on the possible working mechanisms of GlcN in OA. Interference with catabolic activities and stimulation of anabolic activities on chondrocytes have both been reported for GlcN *in vitro*. Studies investigating the influence of GlcN on enzymatic extra-cellular matrix breakdown of cartilage, with a culture system using interleukin-1, lipopolysaccharide or retinoic acid to induce catabolic effects, reported less matrix metalloproteinase activity and aggrecanase activity by addition of GlcN [8-13]. Studies considering the possible anabolic effects of GlcN proposed that addition of GlcN led to more glycosaminoglycan production by chondrocytes, since GlcN is the basic building block of glycosaminoglycan molecules [9,14-17]. These studies were performed with chondrocytes of different species.

Being a rate-limiting precursor in HA synthesis, GlcN was suggested earlier to exert its effects in OA influencing synovial HA synthesis [18,19]. However, direct scientific proof for this was still absent. To our knowledge this is the first study that investigated the effect of GlcN on human OA synovium explants.

In experiment 1 with synovium samples from 6 patients addition of 5 mM GlcN-Ac did not lead to a significant increase in medium HA. Therefore in experiment 2, in which samples from 6 other patients were used, only the effects of the derivative GlcN-HCl were tested at different concentrations and relative to Gluc treatment. In one of our earlier studies using bovine chondrocytes we also found that GlcN-Ac had a different effect on glycosaminoglycan production when compared to GlcN-HCl [20]. We speculate that this difference might be due to the interference of the covalently bound acetyl group as opposed to the ionically bound, and therefore easy to dissolve, hydrochloride salt.

HA is found in almost all tissues, and plays key roles in many cellular processes such as migration, proliferation, differentiation and regulation of matrix organization [21]. HA is one of the main components of synovial fluid in articular joints and plays a major role in joint lubrication and in maintaining joint homeostasis [22]. In inflammatory joints, lower concentration of HA as well as lower molecular weight of HA have been described [23]. Size and concentration of HA determine the viscoelastic properties as well as regulate the inflammatory and tissue repair responses [24,25]. HA was also shown to be able to have anti-inflammatory effects on OA specific cytokines by down-regulation of tumor necrosis factor alpha, interleukin-8 [26]. HA, a polymer of alternating glucuronic acid and GlcN-Ac units is being synthesized at the inside of the cell at the plasma membrane, and then released into the extracellular matrix [27]. In accordance with Recklies et al. our results demonstrate that all 3 isoforms of *HAS*, the enzyme responsible for the synthesis of HA, are expressed by synovium [28]. Recklies et al. observed that the *HAS1* message levels

were always more abundant than that for *HAS2* in synovial cells, and that the *HAS3* message levels were always least abundant. In experiment 2, our mean Ct values showed a similar trend (data not shown). Addition of GlcN-HCl or Gluc did not alter *HAS* gene expression significantly. Thus, direct up-regulation of *HAS* gene expression does not seem to be the explanation for the increased amount of HA in the medium in our experiments. Since *HAS* gene expression was not affected by GlcN, apart from simply providing more building blocks, GlcN could also have played a role in HA synthesis by influencing *HAS* enzymatic activity or protein levels. In the current study design this was not investigated, and therefore this possibility can not be commented on.

The increased amount of HA in the culture medium might be explained by the fact that addition of GlcN-HCl simply led to more building blocks that are required for the synthesis of HA. Our results showed that a concentration of exogenous GlcN ten times lower than the medium Gluc concentration already led to significantly more HA production. This indicated a more effective and perhaps specific effect of exogenous GlcN as opposed to endogenous GlcN that is formed intracellular using medium Gluc as substrate.

The major limitation of our study was the use of relatively high concentrations of GlcN when compared to the *in vivo* situation. A recent report by Persiani et al. [29] showed that synovial GlcN concentration varied between 3 and 18 µM after oral administration at the therapeutic dose of 1500 mg once-a-day. In many earlier reports, including our own, much higher concentration have been used for *in vitro* studies [5,10-13,20,30-34]. In chondrocytes it has been demonstrated that exogenous GlcN was incorporated in newly formed chondroitin sulphate when added at an equimolar concentration with Gluc in the culture medium. When Gluc concentration increased, chondrocytes preferably incorporated GlcN that was endogenously formed from Gluc [35,36]. These results suggested that in chondrocytes the GlcN to Gluc ratio played an important role in the utilization and therefore effectiveness of exogenously provided GlcN. Since we did not know whether this was also the case for synovium, we decided to use an equimolar and ten times lower GlcN concentration compared to medium Gluc concentration.

When trying to translate our *in vitro* results to clinical applicability of GlcN, the intra-articular GlcN concentration that can be reached after administration of GlcN to the patient is reason of concern and thus weakens the direct clinical relevance of our work. Since the first studies were performed on the effect of GlcN there has been debate on this topic. When GlcN-HCl was administered to horses in a single dose, intravenously as well as orally, in a dosage per kg bodyweight at clinically relevant levels, GlcN was still detectable in synovial fluids 6 hours after it was nearly completely cleared from the serum [37]. Although GlcN can thus be retained in joint fluid, it must still be questioned whether the levels of 0.5 mM or 5 mM GlcN will ever be reached in the joint. Therefore, future studies should look into the effect of lower GlcN concentrations on synovial HA production. Our result should be interpreted as a proof of concept on the possible working mechanism of GlcN in OA.

CONCLUSIONS

In conclusion, the results of our experiments suggest that GlcN-HCl increases the production of HA in synovium. Our results were obtained with GlcN concentrations much higher than thus far have been reported in human synovial fluid after oral ingestion of a therapeutic dose [29]. Further studies are needed to investigate the effect of GlcN concentrations more likely to be reached in the human joint, before any decision can be made upon the clinical relevancy of GlcN ingestion on synovial HA production.

REFERENCES

1. Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, et al. Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. N Engl J Med 2006;354:795-808.
2. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, et al. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-6.
3. Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002;162:2113-23.
4. Bellamy N, Campbell J, Robinson V, Gee T, Bourne R, Wells G. Viscosupplementation for the treatment of osteoarthritis of the knee. Cochrane Database Syst Rev 2006:CD005321.
5. Uitterlinden EJ, Jahr H, Koevoet JL, Jenniskens YM, Bierma-Zeinstra SM, Degroot J, et al. Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. Osteoarthritis Cartilage 2006;14:250-7.
6. Mandl EW, Jahr H, Koevoet JL, van Leeuwen JP, Weinans H, Verhaar JA, et al. Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture. Matrix Biol 2004;23:231-41.
7. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.
8. Orth MW, Peters TL, Hawkins JN. Inhibition of articular cartilage degradation by glucosamine-HCl and chondroitin sulphate. Equine Vet J Suppl 2002:224-9.
9. Dodge GR, Jimenez SA. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis Cartilage 2003;11:424-32.
10. Gouze JN, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, et al. Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis: influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. Arthritis Rheum 2001;44:351-60.
11. Sandy JD, Gamett D, Thompson V, Verscharen C. Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. Biochem J 1998;335 (Pt 1):59-66.
12. Fenton JI, Chlebek-Brown KA, Caron JP, Orth MW. Effect of glucosamine on interleukin-1-conditioned articular cartilage. Equine Vet J Suppl 2002:219-23.
13. Byron CR, Orth MW, Venta PJ, Lloyd JW, Caron JP. Influence of glucosamine on matrix metalloproteinase expression and activity in lipopolysaccharide-stimulated equine chondrocytes. Am J Vet Res 2003;64:666-71.
14. Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-34.
15. Bassleer C, Henrotin Y, Franchimont P. In-vitro evaluation of drugs proposed as chondroprotective agents. Int J Tissue React 1992;14:231-41.
16. Lippiello L. Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress. Osteoarthritis Cartilage 2003;11:335-42.
17. Vidal y Plana RR, Bizzarri D, Rovati AL. Articular cartilage pharmacology: I. In vitro studies on glucosamine and non steroidal antiinflammatory drugs. Pharmacol Res Commun 1978;10:557-69.
18. McCarty MF. Enhanced synovial production of hyaluronic acid may explain rapid clinical response to high-dose glucosamine in osteoarthritis. Med Hypotheses 1998;50:507-10.
19. McCarty MF, Russell AL, Seed MP. Sulfated glycosaminoglycans and glucosamine may synergize in promoting synovial hyaluronic acid synthesis. Med Hypotheses 2000;54:798-802.

20. Uitterlinden EJ, Jahr H, Koevoet JL, Bierma-Zeinstra SM, Verhaar JA, Weinans H, et al. Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate. Osteoarthritis Cartilage 2007;15:1267-74.
21. Laurent TC, Fraser JR. Hyaluronan. Faseb J 1992;6:2397-404.
22. Laurent TC, Laurent UB, Fraser JR. The structure and function of hyaluronan: An overview. Immunol Cell Biol 1996;74:A1-7.
23. Dahl LB, Dahl IM, Engstrom-Laurent A, Granath K. Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies. Ann Rheum Dis 1985;44:817-22.
24. Fam H, Bryant JT, Kontopoulou M. Rheological properties of synovial fluids. Biorheology 2007;44: 59-74.
25. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 2007;23: 435-61.
26. Wang CT, Lin YT, Chiang BL, Lin YH, Hou SM. High molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis. Osteoarthritis Cartilage 2006;14:1237-47.
27. Prehm P. Hyaluronate is synthesized at plasma membranes. Biochem J 1984;220:597-600.
28. Recklies AD, White C, Melching L, Roughley PJ. Differential regulation and expression of hyaluronan synthases in human articular chondrocytes, synovial cells and osteosarcoma cells. Biochem J 2001; 354:17-24.
29. Persiani S, Rotini R, Trisolino G, Rovati LC, Locatelli M, Paganini D, et al. Synovial and plasma glucosamine concentrations in osteoarthritic patients following oral crystalline glucosamine sulphate at therapeutic dose. Osteoarthritis Cartilage 2007;15:764-72.
30. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:444-51.
31. de Mattei M, Pellati A, Pasello M, de Terlizzi F, Massari L, Gemmati D, et al. High doses of glucosamine-HCl have detrimental effects on bovine articular cartilage explants cultured in vitro. Osteoarthritis Cartilage 2002;10:816-25.
32. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:258-65.
33. Gouze JN, Bianchi A, Becuwe P, Dauca M, Netter P, Magdalou J, et al. Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-kappa B pathway. FEBS Lett 2002;510:166-70.
34. Mello DM, Nielsen BD, Peters TL, Caron JP, Orth MW. Comparison of inhibitory effects of glucosamine and mannosamine on bovine articular cartilage degradation in vitro. Am J Vet Res 2004;65:1440-5.
35. Mroz PJ, Silbert JE. Effects of [3H]glucosamine concentration on [3H]chondroitin sulphate formation by cultured chondrocytes. Biochem J 2003;376:511-5.
36. Mroz PJ, Silbert JE. Use of (3)H-glucosamine and (35)S-sulfate with cultured human chondrocytes to determine the effect of glucosamine concentration on formation of chondroitin sulfate. Arthritis Rheum 2004;50:3574-9.
37. Laverty S, Sandy JD, Celeste C, Vachon P, Marier JF, Plaas AH. Synovial fluid levels and serum pharmacokinetics in a large animal model following treatment with oral glucosamine at clinically relevant doses. Arthritis Rheum 2005;52:181-91.

Chapter 5

Effect of glucosamine sulphate on joint space narrowing, pain and function in patients with hip osteoarthritis; subgroup analyses of a randomized controlled trial

R.M. Rozendaal, E.J. Uitterlinden, G.J.V.M. van Osch, E.H. Garling, S.P. Willemsen, A.Z. Ginai, J.A.N. Verhaar, H. Weinans, B.W. Koes, S.M.A. Bierma-Zeinstra

Osteoarthritis & Cartilage 2009 Apr; 17(4):427-32

ABSTRACT

Objective

Recently we reported that glucosamine sulphate (GlcN-S) did not have an effect on the symptoms and progression of primary care patients with hip osteoarthritis (OA). The aim of this present study was to investigate whether there are subgroups of patients with hip OA for whom GlcN-S might be an effective therapy.

Method

We randomized 222 patients with hip OA that met one of the American College of Rheumatology criteria to either 1500 mg of oral GlcN-S or placebo once daily for 2 years. Subgroup analyses were predefined for radiographic severity (Kellgren & Lawrence (KL) = 1 vs ≥ 2) and for type of OA (localised vs generalised). Additional exploratory subgroup analyses focused on groups based on pain level, pain medication use, baseline joint space width (JSW), and concomitant knee OA at baseline.

Primary outcome measures were Western Ontario McMaster Universities (WOMAC) pain and function scores over 24 months, and joint space narrowing (JSN) after 24 months.

Results

In the predefined subgroups based on radiographic severity and type of OA, the outcomes WOMAC pain, function and JSN were similar for the GlcN-S and placebo group.

Conclusion

GlcN-S was not significantly better than placebo in reducing symptoms and progression of hip OA in subgroups of patients.

INTRODUCTION

The effect of glucosamine on the symptoms caused by osteoarthritis (OA) and the progression of the disease is still questionable. The summary effect of glucosamine on pain and progression of the disease calculated from the available randomized controlled trials (RCTs) is in favor of glucosamine [1,2]. The individual results of the trials however differ greatly.

Part of the differences in outcome between different trials may be explained by the compound used. A recent review [2] concluded that glucosamine hydrochloride, as used by GAIT [3] and Messier et al. [4], was not effective on OA symptoms, whereas for glucosamine sulphate (GlcN-S) more favorable results are reported. Furthermore, it was suggested that differences in enrolled subjects, outcomes evaluated, and degree of co-intervention could be factors explaining at least some of the differences [5].

Our recently published independent long-term double-blind RCT was the first study to test the effect of GlcN-S in patients with hip OA [6]. The results from this trial showed that GlcN-S has no effect on the symptoms and radiographic progression of hip OA in primary care patients.

When our trial was designed in 2003, not all trials on glucosamine were uniformly positive[7]. Therefore, we hypothesized that GlcN-S might show to be effective only for a subgroup of OA patients. Severity of radiographic OA has previously been suggested to have an influence on the effect of GlcN-S [8,9]. Furthermore, it has been suggested that generalized OA may have a different pathophysiology than localized OA. We therefore hypothesized that type of OA could have an influence on the effect of GlcN-S [10].

In this study we perform predefined and additional exploratory subgroup analyses on the data of the original trial to assess whether there are subgroups of patients with hip OA for whom GlcN-S might be effective in modifying symptomatic and radiographic progression.

METHODS

Design overview

Data of our original study were used for subgroup analyses. This study was a randomized blinded placebo-controlled trial with a duration of 2 years in patients with hip OA [6]. All outcome assessors, patients and researchers in this trial were blinded to therapy.

In the design of the study, a few of the subgroup analyses to assess the effect of GlcN-S in subgroups of patients were pre-specified. The Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam, the Netherlands, approved the study design [MEC226.868/2003/72], and all patients provided written informed consent. A detailed study protocol was published in 2005 [11]. A brief summary of the protocol is presented below.

Setting and participants

General practitioners in the Rotterdam area recruited patients. Patients were eligible for inclusion when they met one of the criteria of the American College of Rheumatology for hip OA [12]. This was screened at the research centre. Patients that had undergone, or were on the waiting list for hip replacement surgery were not included in the study. Patients were excluded when they had a Kellgren & Lawrence (KL) score of 4 [13], renal and/or hepatic disease, diabetes mellitus or disabling co-morbidity. Patients already taking GlcN-S, and those unable to fill out Dutch questionnaires were also excluded.

Patients that violated study protocol or those who underwent total hip arthroplasty were encouraged to stay in the study and fill out questionnaires to limit the loss to follow-up.

For the original study we randomly assigned 222 patients with hip OA to either 1500 mg of GlcN-S once daily (in total 2000 mg of D-glucosamine sulphate 2 Potassium Chloride) or a placebo for the duration of 2 years. Both compounds were manufactured by Nutricia Manufacturing USA, Inc. (Greenville, South Carolina, United States of America) and provided by Numico Research BV (Wageningen, the Netherlands).

The randomization was stratified to allow analyses in subgroups of patients. Patients were stratified on the basis of KL score (1 vs ≥ 2) and on type of OA (localized vs generalized OA). Patients were considered to have generalized OA when they had concomitant radiographic hand and/or knee OA, defined as a KL score of 2 or more. Patients with only OA of the hip joint(s) were classified as having localized OA.

Besides the predefined subgroups based on severity of radiographic OA and type of OA, patients were also divided into groups based on baseline level of pain assessed by a visual analogue scale (VAS) (≤ 30 vs > 30) and on joint space width (JSW) at baseline (≥ 2.5 mm vs < 2.5 mm) for exploratory analyses [14]. We also looked at the effect of treatment in a subgroup of patients that did not use pain medication following the baseline measurement. Finally we assessed whether there was an effect of GlcN-S in a subgroup that had radiographic concomitant knee OA (KL ≥ 2) at baseline.

Outcome measures

For the subgroup analyses we used the data from the primary outcome measures that were used in the original study. These measures were Western Ontario McMaster Universities (WOMAC) pain and function scores over 24 months [15,16] and joint space narrowing (JSN) after 24 months. For the exploratory analyses we only report on the outcomes for WOMAC pain and JSN.

At baseline and after 24 months a highly standardized protocol was used to make weight bearing, anteroposterior digital radiographs of the pelvis, to allow reliable measurement of JSN. At baseline radiography of the hands and knees was performed to assess KL score in these joints. The minimal JSW was measured manually on our digital radiographs, using a computer program (QBone Planner 5.4, manufactured by Medis, Leiden, the Netherlands).

The WOMAC (5-point Likert format), VAS for pain in past week (0-100, 0 equals no pain), and pain medication (type of pain medication and frequency of use: never, occasional, and daily use) were assessed with a questionnaire that was filled out at baseline and every 3 months thereafter; the last at the end of the study after 24 months. The WOMAC subscales are presented as normalized scores (0-100, 0 equals no complaints).

In the case of bilateral hip complaints, patients were asked to indicate their most affected hip. This hip was used for the analyses of JSN. For patients with equivalent pain in both hips, the hip with the highest KL score was used, and in case the KL score was equal as well, the hip with smallest internal rotation during a physical exam was used.

Statistical analysis

For the statistical analyses we used the data of all 3-monthly questionnaires (i.e., baseline, and at 3, 6, 9, 12, 15, 18, 21, and 24 months). The outcomes are given as a mean effect over 24 months incorporating all scores.

The analyses were performed with SPSS 11.0.1 (SPSS inc., Chicago, IL) and SAS 8.2 (SAS Institute, Inc., Cary, NC). The data were analyzed using linear mixed model analyses, under the assumption that data are missing at random. We chose an unstructured covariance structure as this yielded the lowest Akaike's information criterion. Fixed effects were: time, time x therapy, and the covariates we adjusted for. Our model did not have random effects.

For patients who had total hip arthroplasty during the trial, observed data before surgery were included in the analysis and data after surgery were assumed missing. For patients who were lost to follow-up, all observed data were included in the analysis. The analyses for WOMAC and VAS pain were adjusted for factors that may have had an influence on symptoms, being body mass index, gender, and age [17,18]. Analyses were also adjusted for pain medication use and unilateral/bilateral disease as these factors were not fully balanced at baseline, and for KL score. The analyses for JSN were adjusted for KL score [19], age and gender [20].

A total of 24 subgroup analyses were performed of which 20 were reported in the results section, on the basis of this number at least one statistically significant test ($P < 0.05$) can be expected on the basis of chance alone.

RESULTS

In the original study 222 patients were included and randomized to either GlcN-S or placebo. The two groups were similar in baseline and demographic characteristics (Table 1), with the exception of an imbalance in daily pain medication use and unilateral/bilateral disease. As stated before these two factors will be adjusted for in the analyses. The mean values of the outcome measures at baseline for the different subgroups are given in Table 2.

Table 1: Patient characteristics at baseline by randomized group

Characteristics	All randomized patients (n = 222)	Patients randomized to placebo (n = 111)	Patients randomized to GlcN-S* (n = 111)
Women, %	69.4	70.3	68.5
Age in years, mean (SD†)	63.4 (9.0)	63.7 (8.5)	63.1 (9.5)
Body mass index, mean (SD)	28 (4.7)	28 (4.9)	27.9 (4.5)
Duration of complaints < 1 yr, %	11.7	10.8	12.6
1 – 3 yrs, %	34.7	34.2	35.1
> 3 yrs, %	53.6	55	52.3
Localised OA‡, %	38.3	37.8	38.7
Generalised OA, %	61.7	62.2	61.3
Knee OA, %	30.6	30.6	30.6
Unilateral hip OA, %	51.8	38.7	64.9
Bilateral hip OA, %	48.2	61.3	35.1
Kellgren & Lawrence = 1, %	52.7	52.3	53.2
≥ 2, %	47.3	47.7	46.8
Minimum JSW§ in mm, mean (SD)	2.23 (1.0)	2.33 (0.9)	2.13 (1.0)
JSW < 2.5 mm, %	53.6	48.6	58.6
WOMAC¶ pain, mean (SD)	34.2 (23.1)	32.4 (23.2)	35.9 (23.0)
function, mean (SD)	35.1 (22.9)	34.1 (21.7)	36.0 (24.1)
stiffness, mean (SD)	42.6 (25.2)	41.1 (23.1)	44.2 (27.2)
Pain last week in mm, mean (SD)	32.4 (25.9)	30.5 (25.2)	34.3 (26.5)
VAS pain > 30, %	45.0	39.6	50.5
Pain medication use daily, %	23.9	18.9	28.8
sometimes, %	26.6	27.9	25.2
none, %	49.5	53.2	46.0

* GlcN-S: glucosamine sulfate; † SD: standard deviation; ‡ OA: osteoarthritis; § JSW: joint space width; ¶ WOMAC: Western Ontario McMaster Universities

Table 2: Descriptive statistics at baseline for the different subgroups

	WOMAC pain GlcN-S group	WOMAC pain Placebo group	WOMAC function GlcN-S group	WOMAC function Placebo group	JSN* GlcN-S group	JSN Placebo group
KL = 1	32.8 (23.3)	33.7 (24.7)	31.2 (23.6)	34.3 (20.6)	-0.09 (0.36)	0.01 (0.29)
KL ≥ 2	39.5 (22.3)	30.9 (21.7)	41.4 (23.7)	33.9 (22.9)	-0.10 (0.26)	-0.14 (0.35)
Localized OA	34.8 (24.4)	27.3 (20.7)	35.3 (23.6)	29.0 (18.8)	-0.07 (0.30)	-0.07 (0.34)
Generalized OA	36.7 (22.2)	35.5 (24.3)	36.5 (24.6)	37.2 (22.8)	-0.11 (0.35)	-0.05 (0.31)
VAS ≤ 30	20.7 (16.3)	21.0 (14.9)				
VAS > 30	50.9 (18.4)	49.7 (23.0)				
No pain medication	24.0 (20.4)	22.3 (20.0)				
Pain medication	43.7 (21.1)	41.5 (22.4)				
No knee OA	35.2 (23.7)	29.8 (22.8)				
Knee OA	37.5 (21.7)	38.2 (23.4)				
JSW ≥ 2.5 mm					-0.08 (0.34)	0.02 (0.31)
JSW < 2.5 mm					-0.11 (0.32)	-0.15 (0.31)

Values are presented as mean (SD); * JSN: Joint Space Narrowing

Severity of radiographic OA

In an adjusted analysis, the mean difference in WOMAC pain scores over 24 months between GlcN-S and placebo in the group with KL = 1 was -1.4 (95% CI [-6.7, 3.8]) in favor of GlcN-S (Table 3). In the more severe group with KL ≥ 2 the mean difference was -1.4 (95% CI [-7.4, 4.7]). For WOMAC function the mean difference between GlcN-S and placebo in the group with KL = 1 was -1.5 (95% CI [-5.8, 2.9]) whereas this difference was -2.1 (95% CI [-7.5, 3.3]) in the group with KL ≥ 2. For the group with KL = 1 the mean difference in JSN between GlcN-S and placebo was -0.09 mm (95% CI [-0.22, 0.04]), in favor of placebo. In the more severe group (KL ≥ 2) this difference was 0.09 mm (95% CI [-0.07, 0.24]) in favor of GlcN-S.

Table 3: Effect of GlcN-S in subgroups

		Change from baseline after 24 months (SD) Placebo group	Change from baseline after 24 months (SD) GlcN-S group	Unadjusted difference after 24 months (95% CI)	Adjusted difference over 24 months (95% CI)
Predefined subgroup analyses					
WOMAC pain*	KL = 1	-1.5 (26.0)	-4.7 (19.3)	-3.3 (-11.9, 5.4)	-1.4 (-6.7, 3.8)‡
	KL ≥ 2	3.8 (26.8)	3.6 (21.8)	-0.2 (-11.3, 10.9)	-1.35 (-7.4, 4.7)‡
WOMAC function*	KL = 1	-1.0 (18.1)	-2.6 (19.0)	-1.6 (-8.6, 5.5)	-1.5 (-5.8, 2.9)‡
	KL ≥ 2	5.6 (21.3)	1.9 (19.3)	-3.7 (-12.8, 5.5)	-2.1 (-7.5, 3.3)‡
JSN†	KL = 1	0.01 (0.29)	-0.09 (0.36)	-0.10 (-0.22, 0.03)	-0.09 (-0.22, 0.04)§
	KL ≥ 2	-0.14 (0.35)	-0.10 (0.26)	0.03 (-0.11, 0.17)	0.09 (-0.07, 0.24)§
WOMAC pain*	localized	1.8 (31.2)	-1.1 (20.4)	-2.9 (-14.5, 8.8)	1.4 (-5.6, 8.3)‡
	generalized	0.3 (22.8)	-1.8 (21.1)	-2.1 (-10.4, 6.3)	-3.5 (-8.2, 1.3)‡
WOMAC function*	localized	1.2 (23.0)	-2.2 (20.2)	-3.4 (-13.0, 6.2)	-0.9 (-6.9, 5.1)‡
	generalized	2.4 (17.3)	0.3 (18.3)	-2.2 (-9.0, 4.7)	-2.9 (-7.0, 1.2)‡
JSN†	localized	-0.07 (0.34)	-0.07 (0.30)	-0.00 (-0.14, 0.14)	-0.02 (-0.17, 0.13)§
	generalized	-0.05 (0.31)	-0.12 (0.35)	-0.07 (-0.19, 0.06)	-0.05 (-0.18, 0.08)§
Exploratory analyses					
WOMAC pain*	VAS ≤ 30	6.1 (23.7)	1.1 (21.0)	-5.0 (-13.5, 3.5)	-2.4 (-7.1, 2.4)‡
	VAS > 30	-8.0 (28.5)	-4.5 (20.1)	3.5 (-7.5, 14.5)	-3.4 (-10.0, 3.2)‡
WOMAC pain	No pain medication	4.0 (23.6)	1.1 (19.7)	-2.9 (-12.3, 6.4))	-0.3 (-6.4, 5.8)‡
	Pain medication	-2.0 (28.5)	-3.6 (21.3)	-1.6 (-11.5, 8.3)	-3.0 (-8.4, 2.5)‡
WOMAC pain	No knee OA	0.1 (26.2)	0.3 (21.5)	0.3 (-7.9, 8.5)	-0.1 (-4.9, 4.7)‡
	Knee OA	2.9 (27.1)	-5.8 (18.1)	-8.7 (-21.2, 3.8)	-5.7 (-12.6, 1.3)‡
JSN†	≥ 2.5 mm	0.02 (0.31)	-0.08 (0.34)	-0.10 (-0.23, 0.03)	-0.12 (-0.26, 0.01)§
	< 2.5 mm	-0.15 (0.31)	-0.11 (0.32)	0.04 (-0.09, 0.17)	0.04 (-0.09, 0.18)§

* negative value: GlcN-S favours placebo; † positive value: GlcN-S favours placebo; ‡ adjusted for body mass index, gender, age, pain medication use, unilateral/bilateral disease, and Kellgren & Lawrence score; § adjusted for gender, age, and Kellgren & Lawrence score

Type of OA

In the adjusted analysis the mean difference for WOMAC pain in the group with localized OA was 1.4 (95% CI [-5.6, 8.3]) in favor of placebo whereas the mean difference in the generalized subgroup was -3.5 (95% CI [-8.2, 1.3]) in favor of GlcN-S (Table 3). For WOMAC function the adjusted analyses yielded a difference of -0.9 (95% CI [-6.9, 5.1]) for localized OA and -2.9 (95% CI [-7.0, 1.2]) for generalized OA. The mean difference in JSN was -0.02 (95% CI [-0.17, 0.13]) for the localized OA group and -0.05 (95% CI [-0.18, 0.08]) for the generalized OA group, both in favor of the placebo group.

Exploratory analyses

In the group with a pain score at baseline of VAS ≤ 30, the mean difference in WOMAC pain score was -2.4 (95% CI [-7.1, 2.4]) (Table 3). For the group with a pain score at baseline of VAS > 30 the adjusted difference was -3.4 (95% CI [-10.0, 3.2]) in favor of the GlcN-S group.

For patients not using pain medication during the study the adjusted difference in WOMAC pain was -0.3 (95% CI [-6.4, 5.8]). For patients that used pain medication the adjusted difference was -3.0 (95% CI [-8.4, 2.5]).

For patients that had concomitant knee OA at baseline the adjusted difference for WOMAC pain was -5.7 (95% CI [-12.6, 1.3]), while for patients that did not have concomitant knee OA the difference was -0.1 (95% CI [-4.9, 4.7]). In the subgroup with a JSW of more than 2.5 mm at baseline the adjusted difference in JSN was -0.12 (95% CI [-0.26, 0.01]) in favor of placebo. In the subgroup with a JSW of less than 2.5 mm at baseline the adjusted difference in JSN is 0.04 (95% CI [-0.09, 0.18]) and is more in favor of GlcN-S.

DISCUSSION

Subgroup analyses in our trial did not show an effect of GlcN-S on symptomatic or radiographic progression in subgroups of patients with hip OA. The predefined subgroup analyses based on radiographic severity of OA and type of OA did not yield differences between GlcN-S and placebo in outcomes for pain, function and JSN over 24 months. None of the exploratory analyses based on pain medication use, baseline pain level and JSW at baseline showed any differences.

To get a sense of the magnitude of the effects we can express the results for WOMAC pain and function as effect sizes (ES). In order to reach a clinically relevant effect of ES ≥ 0.5, we would need a difference of 11.5 points on WOMAC pain and function outcomes. The ES of all trials on glucosamine is 0.35 [2], which can be interpreted as a small effect. The average lower bound of the confidence intervals in our subgroup analyses was around -7, consistent with an effect size of 0.3. This means that, despite the wider confidence intervals found in subgroup analyses, we can practically rule out even a small effect of GlcN-S.

The possible positive effect of GlcN-S in patients with knee OA [1] raised the question whether the patients within our trial with concomitant knee OA benefited more from GlcN-S then the patients without. We hypothesized that it would be possible to find such an effect in our trial, because complaints due to knee OA are likely to have an influence on the WOMAC questions for hip pain, in particular the ones regarding walking, ascending and descending stairs, and standing.

In the exploratory analysis on the patients with concomitant radiographic knee OA at baseline, we found a mean effect on WOMAC pain of -5.68 (95% CI [-12.62, 1.26]) in favor of GlcN-S whereas the effect is almost 0 in patients without concomitant knee OA. The ES for the lower bound of the confidence interval is 0.56 in favor of GlcN-S, this means we cannot rule out a clinically relevant effect for knee OA.

This difference in effect between hip and knee OA may be due to a difference in inflammatory component, which is thought to be larger in knee OA. This assumption is supported by the finding that some interventions targeting inflammation seem to be more effective for knee than for hip OA [21,22]. Also, in a study by Meulenbelt et al. [23] knee OA was positively associated with the inflammation component, whereas hip OA was not.

That glucosamine acts on the inflammatory processes was already shown by Uitterlinden et al. [24], who found that glucosamine protects against Interleukin-1β (IL-1β) mediated extracellular matrix breakdown in *in vitro* studies. IL-1β is one of the cytokines that plays an important role in the inflammatory cascade in OA [25].

However, the finding for knee OA may also be based on chance alone, due to the multiple tests that we have performed. Also, there is a chance of residual confounding, especially since we did not have other information concerning knee OA than a radiograph of the knees at baseline.

The patients in the original study had milder radiographic OA than patients in previous positive trials [26-28]. While other studies included only patients with KL 2 and 3, about 50% of the patients in this study had a KL score of 1. However, when the effect of GlcN-S in a subgroup of patients with KL score of 2 and 3 was tested, we still found no effect. The outcomes in this subgroup were very similar to the outcomes of the overall study population [6] for WOMAC pain and function.

In addition we performed a subgroup analysis based on JSW, an objective measure of radiographic severity. With a chosen cut-off value of 2.5 mm based on the literature [29], no effect of GlcN-S was seen in the two subgroups.

As far as we know we are the first study to investigate the relationship between type of OA and effect of a therapy. It has been hypothesized that generalized OA may have a different pathophysiology than localized OA [10], which could result in a different reaction to therapy. The results from this current study do not necessarily support this assumption, but they have implications for other studies that study therapeutic effects on a single joint, in which patients

are included with OA in other joints as well. The influence of co-existing disease in other joints should be further investigated.

By performing predefined and exploratory subgroup analyses we increased the possibility of chance findings, the results from our analyses should therefore be interpreted with caution. We chose not to adjust for multiple comparisons. The exploratory analyses were performed to answer questions that emerged during the main analyses, not to draw conclusions on. Adjusting for multiple comparisons would have increased the risk for a type II error. Finally, we found no significant differences without adjusting for multiple comparisons, adjusting for it would have led to even smaller ES.

For the sake of clarity we chose to report only the outcomes for WOMAC pain in the exploratory subgroup analyses. The outcomes for WOMAC function were very similar.

We made subgroups based on baseline level of VAS pain with a cut-off value of 30 as this is often used in literature. In this analysis no differences were found between GlcN-S and placebo. In the Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) study however, a subgroup of patients with a high baseline level of pain (WOMAC pain = 301-400 mm) was found to benefit from the combination of glucosamine hydrochloride and chondroitin sulphate [3]. We did not have enough patients with a level of pain similar to the subgroup in the GAIT study to perform a meaningful analysis on.

The patients in our study were receiving usual care during the study. As a result, half of our patients used pain medication daily or occasionally, as prescribed by their general practitioner or as over-the-counter medication. To rule out a dilution of effect due to pain medication use, we assessed the effect of GlcN-S in a subgroup of patients that reported no use of pain medication throughout the study period. In this group with mild OA complaints (WOMAC pain = 23.1 ± 20.1 (Mean ± SD)) there was still no effect of GlcN-S on pain and function.

In the design of this study subgroup analyses were predefined and the trial was oversized in order to have sufficient power to perform these analyses. However, the standard deviation of the WOMAC pain and function data in our study is much higher than was anticipated, probably due to the inclusion of primary care patients. Future trials with primary care patients should take this into consideration when calculating the sample size. However, despite the large standard deviations in our trial, we were able to rule out minimal clinical important differences in almost all analyses.

CONCLUSION

GlcN-S was not better than placebo in reducing symptoms and progression of hip OA in subgroups of patients based on severity of radiographic OA, type of OA, severity of pain at baseline, pain medication use and baseline JSW.

REFERENCES

1. Towheed T, Maxwell L, Anastassiades T, Shea B, Houpt J, Robinson V, *et al.* Glucosamine therapy for treating osteoarthritis. Cochrane Database Syst Rev 2005:CD002946.
2. Vlad SC, LaValley MP, McAlindon TE, Felson DT. Glucosamine for pain in osteoarthritis: why do trial results differ? Arthritis Rheum 2007;56:2267-77.
3. Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, *et al.* Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. N Engl J Med 2006;354:795-808.
4. Messier SP, Mihalko S, Loeser RF, Legault C, Jolla J, Pfruender J, *et al.* Glucosamine/chondroitin combined with exercise for the treatment of knee osteoarthritis: a preliminary study. Osteoarthritis Cartilage 2007;15:1256-66.
5. Towheed TE, Anastassiades T. Glucosamine therapy for osteoarthritis: an update. J Rheumatol 2007; 34:1787-90.
6. Rozendaal RM, Koes BW, van Osch GJ, Uitterlinden EJ, Garling EH, Willemsen SP, *et al.* Effect of glucosamine sulfate on hip osteoarthritis: a randomized trial. Ann Intern Med 2008;148:268-77.
7. McAlindon T. Why are clinical trials of glucosamine no longer uniformly positive? Rheum Dis Clin North Am 2003;29:789-801.
8. Reginster JY, Bruyere O, Neuprez A. Current role of glucosamine in the treatment of osteoarthritis. Rheumatology (Oxford) 2007;46:731-5.
9. Rindone JP, Hiller D, Collacott E, Nordhaugen N, Arriola G. Randomized, controlled trial of glucosamine for treating osteoarthritis of the knee. West J Med 2000;172:91-4.
10. Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, *et al.* Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 2000;133:635-46.
11. Rozendaal RM, Koes BW, Weinans H, Uitterlinden EJ, van Osch GJ, Ginai AZ, *et al.* The effect of glucosamine sulphate on osteoarthritis: design of a long-term randomised clinical trial [ISRCTN54513166]. BMC Musculoskelet Disord 2005;6:20.
12. Altman R, Alarcon G, Appelrouth D, Bloch D, Borenstein D, Brandt K, *et al.* The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. Arthritis Rheum 1991;34:505-14.
13. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494-502.
14. Croft P, Cooper C, Coggon D. Case definition of hip osteoarthritis in epidemiologic studies. J Rheumatol 1994;21:591-2.
15. Bellamy N, Buchanan WW, Goldsmith CH, Campbell J, Stitt LW. Validation study of WOMAC: a health status instrument for measuring clinically important patient relevant outcomes to antirheumatic drug therapy in patients with osteoarthritis of the hip or knee. J Rheumatol 1988;15:1833-40.
16. Roorda LD, Jones CA, Waltz M, Lankhorst GJ, Bouter LM, van der Eijken JW, *et al.* Satisfactory cross cultural equivalence of the Dutch WOMAC in patients with hip osteoarthritis waiting for arthroplasty. Ann Rheum Dis 2004;63:36-42.
17. Peters TJ, Sanders C, Dieppe P, Donovan J. Factors associated with change in pain and disability over time: a community-based prospective observational study of hip and knee osteoarthritis. Br J Gen Pract 2005;55:205-11.
18. van Dijk GM, Dekker J, Veenhof C, van den Ende CH. Course of functional status and pain in osteoarthritis of the hip or knee: a systematic review of the literature. Arthritis Rheum 2006;55:779-85.
19. Reijman M, Hazes JM, Pols HA, Bernsen RM, Koes BW, Bierma-Zeinstra SM. Role of radiography in predicting progression of osteoarthritis of the hip: prospective cohort study. Bmj 2005;330:1183.
20. Lievense AM, Bierma-Zeinstra SM, Verhagen AP, Verhaar JA, Koes BW. Prognostic factors of progress of hip osteoarthritis: a systematic review. Arthritis Rheum 2002;47:556-62.

21. Svensson O, Malmenas M, Fajutrao L, Roos EM, Lohmander LS. Greater reduction of knee than hip pain in osteoarthritis treated with naproxen, as evaluated by WOMAC and SF-36. Ann Rheum Dis 2006;65:781-4.
22. Fidelix TS, Soares BG, Trevisani VF. Diacerein for osteoarthritis. Cochrane Database Syst Rev 2006:CD005117.
23. Meulenbelt I, Kloppenburg M, Kroon HM, Houwing-Duistermaat JJ, Garnero P, Hellio-Le Graverand MP, *et al*. Clusters of biochemical markers are associated with radiographic subtypes of osteoarthritis (OA) in subject with familial OA at multiple sites. The GARP study. Osteoarthritis Cartilage 2007;15: 379-85.
24. Uitterlinden EJ, Jahr H, Koevoet JL, Bierma-Zeinstra SM, Verhaar JA, Weinans H, *et al*. Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate. Osteoarthritis Cartilage 2007;15:1267-74.
25. Fernandes JC, Martel-Pelletier J, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. Biorheology 2002;39:237-46.
26. Herrero-Beaumont G, Ivorra JA, Del Carmen Trabado M, Blanco FJ, Benito P, Martin-Mola E, *et al*. Glucosamine sulfate in the treatment of knee osteoarthritis symptoms: a randomized, double-blind, placebo-controlled study using acetaminophen as a side comparator. Arthritis Rheum 2007;56:555-67.
27. Pavelka K, Bruyere O, Rovati LC, Olejarova M, Giacovelli G, Reginster JY. Relief in mild-to-moderate pain is not a confounder in joint space narrowing assessment of full extension knee radiographs in recent osteoarthritis structure-modifying drug trials. Osteoarthritis Cartilage 2003;11:730-7.
28. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, *et al*. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-6.
29. Croft P, Cooper C, Wickham C, Coggon D. Defining osteoarthritis of the hip for epidemiologic studies. Am J Epidemiol 1990;132:514-22.

Chapter 6

Does oral glucosamine detectably alter gene expression in cartilage and bone? An explorative pilot study in patients with hip osteoarthritis

E.J. Uitterlinden, H. Jahr, R.M. Rozendaal, J.L.M. Koevoet, S.M.A. Bierma-Zeinstra, J.A.N. Verhaar, G.J.V.M. van Osch, H. Weinans

Manuscript in preparation

ABSTRACT

Objective

To explore the effects of pre-operative glucosamine (GlcN) administration on expression of genes involved in anabolic and catabolic activities in human osteoarthritic hip cartilage and subchondral bone.

Methods

Glucosamine-hydrochloride (GlcN-HCl) or glucosamine-sulphate (GlcN-S) was administered orally pre-operative in a once-daily dose of 1500 mg in two placebo controlled studies to patients awaiting total hip replacement surgery. Per-operative cartilage samples (both studies) and bone samples (GlcN-S study) were obtained, RNA was isolated and real time RT-PCR performed. Sampling size calculations were performed based on these data. Per-operatively obtained cartilage samples (GlcN-HCl study) were cultured with and without GlcN-HCl and subsequently gene expression was analyzed.

Results

This explorative study showed indications for down-regulation of aggrecan gene expression in cartilage from patients who used GlcN-HCl when compared to placebo. Gene expression in subchondral bone from patients who used GlcN-S indicated MMP13 down-regulation compared to placebo. Adding GlcN-HCl to *in vitro* cultures of cartilage explants of the GlcN-HCl study indicated down-regulation of both collagen type II and aggrecan gene expression. This effect was more prominent in the cartilage explants from patients who took GlcN-HCl pre-operatively.

Conclusion

It appears possible to detect changes in gene expression after pre-operative oral administration of GlcN. Administration of GlcN-HCl may have primed the cartilage such, that its sensitivity to adding GlcN-HCl *in vitro* increased. The trend towards down-regulation of aggrecan and collagen type II gene expression for GlcN-HCl compared to placebo in this explorative study, should be replicated in future studies. The necessary sampling sizes for such future investigations are provided.

INTRODUCTION

Glucosamine (GlcN) is widely being used by patients suffering from osteoarthritis (OA), yet without severe adverse effects being reported [1]. Most of the evidence concerning the clinical effectiveness of GlcN until now is based on patients with knee OA. Recently a multicenter trial sponsored by the National Institutes of Health called the Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) was conducted [2]. Considering the effects of GlcN on pain, subgroup analysis in this GAIT trial and an ancillary study of the GAIT trial showed that there might be an effect in people with mild to severe OA [2,3]. Earlier X-ray based studies concluded that GlcN delayed disease progression in patients with knee OA during a 3 year period [4,5]. Although the clinical evidence is mostly based on patients with knee OA, GlcN is also being used by patients with OA in joints other than the knee. Whether the effects of GlcN on knee OA also applies to OA in other joints, such as the hip, has been questioned by other authors [6].

Several articles, including those from our group, have shown that GlcN can have an effect on the articular joint cells and tissues involved in OA [7-25]. A limitation of *in vitro* studies on the effects of GlcN on chondrocytes is the use of supraphysiological GlcN concentrations. Most *in vitro* studies use GlcN concentrations in the millimolar range. Silbert found maximum GlcN serum levels in humans of 2-12 μM two to three hours after ingestion of 1500 mg glucosamine-sulphate (GlcN-S) after an overnight fast [26]. In joint fluid, Persiani et al. showed that administration of 1500 mg crystalline GlcN-S once daily for 14 days led to a median post treatment synovial GlcN concentration range of approximately 3-18 μM [27]. One solution to bypass this translation of *in vitro* data to clinical relevance is to administer GlcN to patients in a placebo controlled trial and explore the effect on cartilage that can be obtained at total joint arthroplasty.

The aim of this study was to explore whether changes in gene expression of cartilage and/or subchondral bone can be detected in patients with hip OA after pre-operative oral glucosamine-hydrochloride (GlcN-HCl) or GlcN-S administration when compared to patients treated with a placebo. Since this is an explorative pilot study, we used these collected *in vivo* gene expression data to provide directions for sampling size in future studies investigating effects of GlcN on *in vivo* gene expression. Furthermore we explored whether the pre-operative oral intake of GlcN, primed chondrocytes to alter their response to GlcN in post-operative cartilage explant culture experiments.

MATERIALS AND METHODS

Patient population and pre-operative assessments

GlcN-HCl study

The waiting list for total hip replacement surgery at the Erasmus MC, University Medical Center Rotterdam was periodically screened for patients eligible for participation in the study, according to the in- and exclusion criteria in table 1. If so, they received information about the study at home and got an appointment with the investigator (E.U.) at the same day as their pre-operative outpatient clinic appointment. During this visit, again after checking in- and exclusion criteria, definitive written informed consent was obtained, with approval of the Ethical Committee (MEC 204.287/2001/196). The patients included in the study were randomized by the hospital pharmacist to the GlcN-HCl or placebo group. All investigators as well as the patients were blinded for the randomization result. Randomization was stratified for sex, in order to reach an equal male to female ratio in the GlcN-HCl and placebo group. Block randomization was used in order to secure a proper placebo to GlcN-HCl ratio. The GlcN-HCl and placebo pills were provided by Numico Research B.V. (Wageningen, the Netherlands) but were produced at Nutricia manufacturing USA Inc. (Greenville, SC). Each individual GlcN-HCl pill contained 750 mg GlcN-HCl and patients were instructed to take 2 pills once daily in order to reach a 1500 mg daily dose. Both the GlcN-HCl and placebo pills were identical in smell, appearance and taste and were provided in containers that looked similar. Each patient received 140 pills, which was enough for 10 weeks medication use. At the time of inclusion, pain level was determined using a visual analogue scale (VAS) regarding the pain experienced over the previous week and the pain subscale of the Western Ontario and McMaster Universities index (WOMAC pain) was recorded using a five point Likert format. The VAS was presented on a 10 cm scale, with 0 representing no pain and 10 the worst pain imaginable. The WOMAC pain subscale was presented as a normalized score (0-100, where a score of 0 equals no pain).

Table 1. GlcN-HCl study, in- and exclusion criteria

Inclusion criteria	Exclusion criteria
Idiopathic hip osteoarthritis	Osteonecrosis on major trochator
On waiting list for total hip replacement	Diabetes Mellitus
Older than 50 (male) or 60 (female) and younger than 80 years at time of surgery	Younger than 50 (male) or 60 (female) and older than 80 years at time of surgery
Kellgren and Lawrence grade 2 and 3	Kellgren and Lawrence grade 4
Informed consent	Alcohol or drug abuse
	Use of corticosteroids
	Use of food supplements as glucosamine and/or chondroitinesulphate
	No understanding of Dutch language
	Cognitive impairment

Eight to ten weeks prior to scheduled total hip replacement surgery patients got a phone call of the investigator with the instruction to start taking the pills they received. To improve compliance, patients were contacted by phone four weeks after starting the use of the study medication. At this time the WOMAC pain was recorded by phone again. The day before surgery patients were admitted to the hospital. Now the VAS for pain and the WOMAC pain was assessed again. Patient compliance was monitored using the brief medication questionnaire as published by Svarstad et al. [28]. On the day of surgery, cartilage from the femoral head was obtained for further analysis as is described below.

GlcN-S study

For the exact description of the study set up of the GlcN-S study, we refer to the article by Rozendaal et al. [29]. In short, patients from the general practitioners population in the Rotterdam area with hip OA according to the ACR criteria were recruited to participate in a double blind randomized placebo controlled clinical trial on the effect of GlcN-S on hip OA (MEC 226.868/2003/72). If patients underwent total hip replacement surgery during the course of this study, femoral heads were collected at the time of surgery. Obtaining the femoral heads was only possible after the patients gave their informed consent and if the operating orthopaedic surgeon, mostly in hospitals in the Rotterdam area other than the Erasmus MC, was willing to cooperate in the study. Prior to the moment of operation several measurements were undertaken as can be found in the article by Rozendaal et al. [29]. In this study we only report on the data of the patients whose femoral heads we were able to obtain when they underwent total hip replacement surgery during the 2-year period of the trial. In the GlcN-S study, the largest differences compared to the GlcN-HCl study was the GlcN derivative that was used (GlcN-S instead of GlcN-HCl) and the non standardized duration of study medication use until the moment of surgery. Therefore, in the GlcN-S study we only report the WOMAC pain and VAS score at the start of the study and the last one that had been assessed prior to total hip replacement surgery. On the day of surgery, cartilage and bone samples from the femoral head were obtained for further analysis as is described below.

Tissue harvesting and processing and data analyses

Per-operative cartilage samples, GlcN-HCl study & GlcN-S study

On the day of total hip replacement surgery, the femoral head was obtained as quickly as possible after it was detached from the femoral neck by the orthopaedic surgeon. Under sterile conditions in the operating room, the head was cleansed using gauze with physiological saline to remove any blood or bone marrow spill from the cartilage of the femoral head. Then two slices of macroscopically normal, full thickness cartilage were harvested and snap frozen in liquid nitrogen. The wet weight per sample was determined and the samples were further processed as described in the section on gene expression analysis.

Per-operative bone samples, GlcN-S study

After the removal of the first two slices of cartilage in the operating room, the femoral head was transported to the laboratory under sterile conditions. Using a custom made hollow 8 mm diameter diamond coated drill, one bone plug was drilled from a location with macroscopically normal cartilage still *in situ*. During the drilling process, the bone was protected from overheating by cooling with physiological saline at 4° C in a drop wise fashion. After drilling the bone plug, cartilage was removed and it was divided in one slice of subchondral bone (sb) with a thickness that varied dependent on the subchondral bone sclerosis present, and 5 slices of trabecular bone of 2 mm thickness each, numbered one to five consecutively with five the most far away from the subchondral bone (tb1 - tb5). This was done using a hammer and an osteotome. Directly after the different slices were created, they were snap frozen in liquid nitrogen. The wet weight per sample was determined and the samples were further processed as described in the section on gene expression analysis.

Post-operative cartilage culture experiments, GlcN-HCl study

After removal of the slices of cartilage in the operating room, for analysis of the effect of GlcN on gene expression, explants were taken from macroscopically intact cartilage of the osteoarthritic femoral head, using a 4 mm diameter dermal biopsy punch. Explants were separated from the underlying bone by dissection with a scalpel and pooled in a Petri dish. In these culture experiments we compared two conditions: 0.5 mM GlcN-HCl and control. Per condition, ten explants were randomly taken from the Petri dish and cultured in one well of a 6-well plate with culture medium consisting of 3 ml low glucose (1000mg/l = 5.55 mM) DMEM (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum, 50 µg/ml gentamicin, 1.5 µg/ml fungizone and 25 µg/ml L-ascorbic acid-2-phosphate freshly added, during the whole experiment. After an initial three-day pre-culture period (days 0-3), 0.5 mM GlcN-HCl (Sigma, St Louis, MO) was added for four days (days 3-7). For the control condition no GlcN-HCl was added. Culture medium with or without GlcN-HCl was refreshed once. After a total of seven days of culture the explants were harvested and snap frozen in liquid nitrogen. The wet weight per sample was determined and the samples were further processed as described in the section on gene expression analysis below.

Gene expression analysis

Tissue processing and gene expression analysis were performed using the procedures and protocols as described earlier [23,30]. We explored genes involved in anabolic processes (cartilage: collagen type II (COL2) and aggrecan (AGC1); bone: collagen type I and X, osteopontin and integrin-binding sialoprotein), catabolic processes (cartilage: matrix metalloproteinases, MMP2, 9, 14; bone and cartilage: MMP1, 3, 13 and cartilage: ADAMTS (a desintegrin and a metalloproteinase with thrombospondin motifs, 4 and 5) and regulatory genes (TIMP; tissue inhibitors of MMP, 1 and 2). Glyceraldehyde-3-phosphate dehydrogenase was stably expressed between

the samples within all experiments. The Ct values of the per-operative cartilage and bone samples and for cartilage culture experiment samples were all normalized to glyceraldehyde-3-phosphate dehydrogenase, according to the $2^{-\Delta\Delta Ct}$ method by Livak et al. [31]. To explore any difference in gene expression of the per-operative bone and cartilage samples and the control and the 0.5 mM GlcN-HCl condition between the placebo and GlcN group a Mann-Whitney U test was performed using normalized Ct values (i.e., $2^{-\Delta Ct}$). A Wilcoxon signed ranks test was used to explore any difference in gene expression of the cartilage culture experiments within the placebo or GlcN group using the normalized Ct values. A p-value < 0.05 was considered to indicate possible significant differences. All statistical analysis was performed using SPSS 15.0.1 (SPSS Inc., Chicago, IL).

Sampling size

To establish sampling size, we used the normalized Ct values of the per-operative cartilage samples of the patients who used a placebo in both the GlcN-HCl and GlcN-S study. From these values the mean and the standard deviation was calculated per gene of interest. Using a power of 0.8 ($\beta = 0.2$) and $\alpha = 0.05$, we used the following formula to calculate the required sampling size for one-sided testing needed to reliably detect a ≥ 2-fold difference in expression between GlcN-treated and placebo-treated samples:

$N = 2\{Z_{1-\alpha} + Z_{1-\beta}\}^2 * ((SD_{placebo})^2 / (\Delta\ mean_{placebo/GlcN})^2)$, where $2\{Z_{1-\alpha} + Z_{1-\beta}\}^2$ equals 12.3.

RESULTS

Study populations and the effect of GlcN on symptomatic severity

GlcN-HCl study

We finally managed to obtain the material of eleven patients from the GlcN-HCl study at the time of total hip replacement surgery. Six of them received placebo and 5 received GlcN-HCl (Fig. 1). Details on patient characteristics and the pre-operative assessments of GlcN-HCl study can be found in table 2.

GlcN-S study

From the 222 patients who were enrolled in the study by Rozendaal et al., we were able to collect 15 femoral heads of the 20 patients who underwent total hip replacement surgery. Five of these people received a placebo and ten patients received GlcN-S. These unequal numbers are the result of the fact that a total hip replacement operation was an uncontrolled event in this study. Details on patient characteristics and the pre-operative assessments of GlcN-S study can be found in table 3.

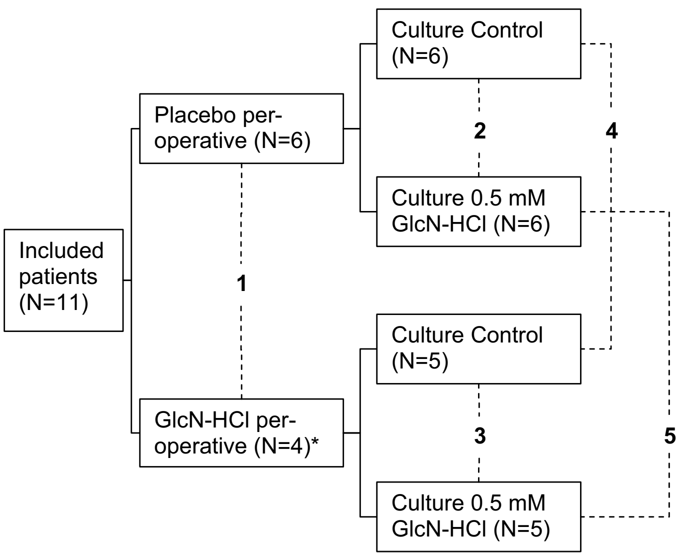

Figure 1. Schematic drawing of the study set-up of gene expression analysis of cartilage in GlcN-HCl study and the number of the patients in each (sub)group. 1: comparison of gene expression of cartilage samples per-operative; 2: comparison of gene expression between 0.5 mM GlcN-HCl and control after culture of samples obtained from patients in the placebo group; 3: comparison of gene expression between 0.5 mM GlcN-HCl and control after culture of samples obtained from patients in the GlcN-HCl group; 4: comparison of gene expression of the control condition after culture between samples obtained from patients in the placebo and in the GlcN-HCl group; 5: comparison of gene expression of the 0.5 mM GlcN-HCl condition after culture between samples obtained from patients in the placebo and in the GlcN-HCl group. * The data of the per-operative cartilage samples of one patient in the GlcN-HCl group are missing, due to technical difficulties during tissue processing and RNA extraction.

Table 2. GlcN-HCl study, patient characteristics and pre-operative assessments

	Placebo (n=6)		GlcN-HCl (n=5)	
M:F ratio	2:4		1:4	
Mean Age (SD), y	68.5	(10.0)	65.8	(5.7)
Days preoperative medication use (SD)	57.2	(0.4)	58.2	(6.1)
Mean WOMAC pain score (SD) §				
inclusion	65.0	(16.7)	59.0	(11.4)
4 weeks after medication start	65.8	(16.6)	64.0	(18.5)
day prior to surgery	64.2	(23.1)	69.0	(18.5)
Mean pain past week VAS (SD), cm				
inclusion	6.3	(1.8)	5.7	(1.7)
day prior to surgery	6.6	(2.8)	6.8	(2.0)

WOMAC = Western Ontario and McMaster Universities; VAS = Visual Analogue Scale; § Score is normalized (0-100; 0 = no pain)

Table 3. GlcN-S study, patient characteristics and pre-operative assessments

	Placebo (n=5)		GlcN-S (n=10)	
M:F ratio	4:1		2:8	
Mean Age (SD), y	66.0	(7.9)	64.3	(8.6)
Days preoperative medication use (SD)	337.4	(182.2)	321.2	(162.7)
Mean WOMAC pain score (SD) §				
inclusion	46.0	(20.7)	56.5	(12.0)
last assessment prior to surgery	59.0	(11.4)	59.0	(24.5)
Mean pain past week VAS (SD), cm				
inclusion	6.3	(3.6)	5.3	(2.7)
last assessment prior to surgery	6.7	(2.2)	6.1	(2.8)

WOMAC = Western Ontario and McMaster Universities; VAS = Visual Analogue Scale; § Score is normalized (0-100; 0 = no pain)

Does oral GlcN intake result in detectable changes in cartilage gene expression *in vivo*?

GlcN-HCl study

The data of the per-operative cartilage samples of one patient in the GlcN-HCl group are missing, due to technical difficulties during tissue processing and RNA extraction. This led to an analysis based on four GlcN-HCl and six placebo per-operative cartilage samples. The mean and standard deviation (SD) of the normalized gene expression of the genes tested in the placebo and GlcN-HCl group can be found in table 4. For the per-operative cartilage samples an indication for a statistically significant down-regulation in aggrecan gene expression was found for the GlcN-HCl group when compared to the placebo group. For all the other tested genes, no statistically significant difference in expression was found between the placebo and GlcN-HCl group.

Table 4. Normalized gene expression in cartilage *in vivo* after oral GlcN-HCl intake

	Pre-operative placebo		Pre-operative GlcN-HCl		
Gene	**Mean**	**SD**	**Mean**	**SD**	***P***
AGC1	3.0290	1.0637	1.4001	0.9702	*0.038*
COL2	13.0260	7.3132	12.8791	12.0003	*0.914*
MMP1	0.0002	0.0004	0.0001	0.0001	*0.762*
MMP2	0.2249	0.2714	0.3500	0.4376	*0.914*
MMP3	0.1088	0.0528	0.1133	0.1111	*0.610*
MMP9	0.0057	0.0097	0.1199	0.2066	*0.352*
MMP13	0.2383	0.2438	0.2411	0.2803	*0.762*
MMP14	0.0704	0.0567	0.0618	0.0540	*0.610*
ADAMTS4	0.0015	0.0018	0.0020	0.0017	*0.610*
ADAMTS5	0.0087	0.0064	0.0093	0.0023	*1.000*
TIMP1	0.0011	0.0007	0.0150	0.0234	*0.257*
TIMP2	0.2117	0.1317	0.2751	0.0640	*0.476*

GlcN-S study

The data of the per-operative cartilage samples of one patient in the placebo group and one patient in the GlcN-S group are missing, due to technical difficulties during tissue processing and RNA extraction, leading to an analysis based on 4 placebo and 9 GlcN-S per-operative cartilage samples. The mean and SD of the normalized gene expression of the genes tested in the placebo and GlcN-S group can be found in table 5. No statistically significant differences between both groups were found for any of the tested genes of the per-operative cartilage samples.

Table 5. Normalized gene expression in cartilage *in vivo* after oral GlcN-S intake

	Pre-operative placebo		**Pre-operative GlcN-S**		
Gene	**Mean**	**SD**	**Mean**	**SD**	***P***
AGC1	1.1242	0.6220	2.2335	1.0463	*0.076*
COL2	12.9679	6.7534	17.1461	8.5509	*0.414*
MMP1	0.0116	0.0164	0.0003	0.0008	*0.667*
MMP2	0.0179	0.0318	0.0980	0.1704	*0.503*
MMP3	0.0117	0.0116	0.0447	0.0727	*0.106*
MMP9	0.0001	0.00002	0.0450	0.1160	*0.327*
MMP13	0.0621	0.0926	0.1239	0.2813	*0.710*
MMP14	0.0288	0.0199	0.0398	0.0682	*0.503*
ADAMTS4	0.0003	- (N=1)	0.0009	0.0019	*0.600*
ADAMTS5	0.0011	0.0008	0.0038	0.0053	*0.482*
TIMP1	0.0001	0.0002	2.2976	6.8925	*0.503*
TIMP2	0.1227	0.0900	0.1276	0.0524	*0.825*

Does oral GlcN-S intake result in detectable changes in bone gene expression *in vivo*?

We analyzed the subchondral bone samples and trabecular bone samples from levels one and three. Although samples from all fifteen patients were available, a fair number of data is missing due to technical difficulties during tissue processing and RNA extraction.

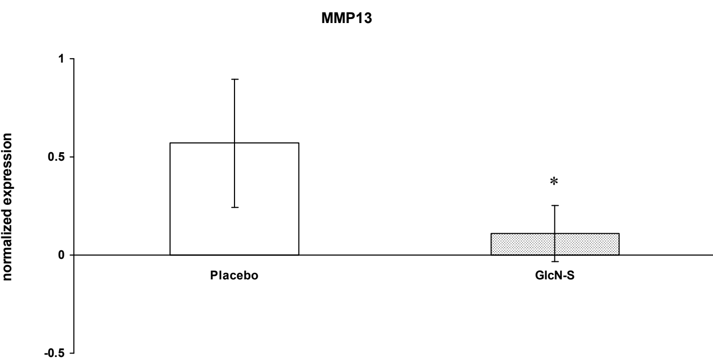

Figure 2. For the GlcN-S study, normalized expression for MMP13 gene expression of per-operative trabecular bone level 3 samples of the placebo (N=3) and GlcN-S (N=8) group are displayed. * indicates a statistically significant difference compared to the placebo group (p-value < 0.05).

Within the data that were available after processing of the bone samples, we explored whether there was a statistically significant difference in gene expression, between the placebo and the GlcN-S group. The only borderline significant difference we found, was a lower expression of MMP13 (= collagenase 3) in the GlcN-S group (N=8) when compared to placebo (N=3) in the tb3 samples ($p = 0.048$, Fig. 2). For all other levels and genes tested, no significant differences were found.

Does oral GlcN-HCl intake influence gene expression changes in culture experiments with GlcN-HCl *in vitro*?

Details on the gene expression data after the culture experiments, that were determined on explants originating from five patients who pre-operatively received GlcN-HCl and six patients who received placebo pre-operatively, can be found in table 6. When exploring the data without considering the pre-operative treatment the cartilage explants were exposed to *in vivo* (Fig. 1; 2 and 3 combined) we found indications for a statistically significant down-regulation after addition of 0.5 mM GlcN-HCl when compared to the control condition for aggrecan (Wilcoxon signed ranks test, $p = 0.033$, Fig. 3 panel A) and collagen type II (Wilcoxon signed ranks test, $p = 0.013$, Fig. 4 panel A) gene expression. When we did consider the pre-operative treatment, gene expression of the cultured explants from the patients who used a placebo pre-operatively (Fig. 1; 2) revealed no indication of a statistically significant difference comparing the 0.5 mM GlcN-HCl condition with the corresponding controls (Fig. 3 and 4 panel B). In the group that used GlcN-HCl pre-operatively (Fig. 1; 3), again indications for a statistically significant down-regulation after addition of 0.5 mM GlcN-HCl were found compared to the control condition,

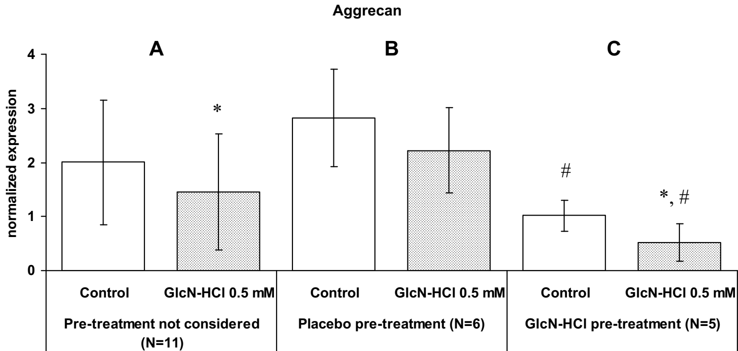

Figure 3. Normalized expression for aggrecan gene expression in cartilage explants using cartilage from patients in the GlcN-HCl study. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 0.5 mM GlcN-HCl or control. Panel A displays the results of all samples combined (thus pre-treatment not considered) and panel B and C display the results of the samples that received placebo or GlcN-HCl pre-treatment respectively. * indicates a statistically significant difference compared to the corresponding control group (Wilcoxon signed ranks test, $p < 0.05$). # indicates a statistically significant difference compared to the same condition in the placebo pre-treatment group (Mann-Whitney U test, $p < 0.05$).

Table 6. Normalized gene expression in cartilage explants after culture experiments (p-values determined using a Wilcoxon signed ranks test)

	Pre-treatment not considered					***Placebo pre-treatment***					***GlcN-HCl pre-treatment***				
	Control		**GlcN-HCl 0.5 mM**			**Control**		**GlcN-HCl 0.5 mM**			**Control**		**GlcN-HCl 0.5 mM**		
Gene	**Mean**	**SD**	**Mean**	**SD**	***P***	**Mean**	**SD**	**Mean**	**SD**	***P***	**Mean**	**SD**	**Mean**	**SD**	***P***
AGC1	2.002	1.154	1.450	1.075	*0.033*	2.826	0.898	2.224	0.796	*0.249*	1.013	0.285	0.522	0.346	*0.043*
COL2	7.430	4.658	2.899	2.325	*0.013*	8.486	3.951	4.623	1.703	*0.075*	6.162	5.571	0.831	0.296	*0.043*
MMP1	0.046	0.024	0.036	0.015	*0.328*	0.032	0.019	0.041	0.014	*0.463*	0.063	0.019	0.030	0.015	*0.080*
MMP2	0.558	0.481	0.665	0.612	*0.722*	0.369	0.397	0.649	0.737	*0.753*	0.784	0.514	0.684	0.505	*0.225*
MMP3	1.803	1.163	1.337	0.667	*0.131*	1.182	0.770	1.167	0.681	*0.753*	2.548	1.169	1.541	0.661	*0.080*
MMP9	0.001	0.001	0.001	0.001	*0.799*	0.001	0.001	0.001	0.001	*0.500*	0.001	0.001	0.001	0.001	*0.686*
MMP13	1.786	1.186	1.974	1.234	*0.182*	2.154	1.232	2.400	0.928	*0.249*	1.345	1.081	1.461	1.459	*0.893*
MMP14	0.398	0.219	0.475	0.354	*0.534*	0.330	0.157	0.408	0.345	*0.753*	0.479	0.272	0.555	0.386	*0.500*
ADAMTS4	0.002	0.002	0.002	0.001	*0.286*	0.001	0.001	0.001	0.001	*0.753*	0.003	0.002	0.002	0.002	*0.080*
ADAMTS5	0.007	0.005	0.010	0.009	*0.424*	0.007	0.006	0.011	0.010	*0.116*	0.006	0.004	0.009	0.009	*0.686*
TIMP1	0.007	0.007	0.010	0.015	*0.424*	0.005	0.003	0.004	0.002	*0.116*	0.008	0.011	0.017	0.021	*0.500*
TIMP2	0.532	0.149	0.520	0.160	*0.722*	0.584	0.111	0.604	0.147	*0.917*	0.471	0.177	0.420	0.120	*0.500*

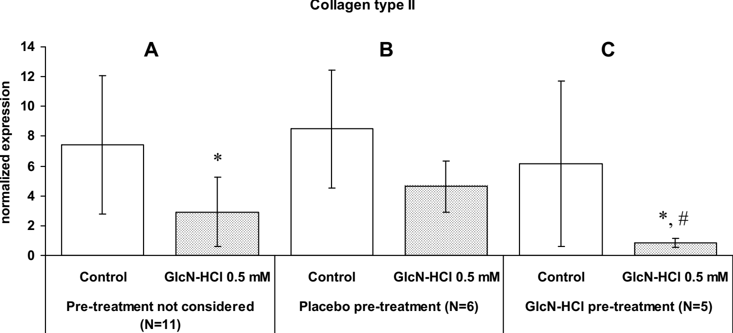

Figure 4. Normalized expression for collagen type II gene expression in cartilage explants using cartilage from patients in the GlcN-HCl study. Cartilage explants were pre-cultured for 3 days, followed by 4 days of treatment with 0.5 mM GlcN-HCl or control. Panel A displays the results of all samples combined (thus pre-treatment not considered) and panel B and C display the results of the samples that received placebo or GlcN-HCl pre-treatment respectively. * indicates a statistically significant difference compared to the corresponding control group (Wilcoxon signed ranks test, $p < 0.05$). # indicates a statistically significant difference compared to the same condition in the placebo pre-treatment group (Mann-Whitney U test, $p < 0.05$).

both for aggrecan (Wilcoxon signed ranks test, p = 0.043, Fig. 3 panel C) and collagen type II (Wilcoxon signed ranks test p = 0.043, Fig. 4 panel C) gene expression.

Finally, we explored whether aggrecan and collagen type II gene expression were different between the control condition of the placebo pre-treatment samples and the control condition of the GlcN-HCl pre-treatment samples (Fig. 1; 4).This was also determined for the 0.5 mM GlcN-HCl condition (Fig. 1; 5). For aggrecan gene expression we found indications of a statistically significant lower gene expression of both the control (Mann-Whitney U test, p= 0.004) and 0.5 mM GlcN-HCl condition (Mann-Whitney U test, p= 0.004) for the GlcN-HCl pre-treatment samples, when compared to the placebo pre-treatment samples (Fig. 3, panel B and C). For collagen type II gene expression we found indications of a statistically significant lower gene expression of the 0.5 mM GlcN-HCl condition (Mann-Whitney U test, p= 0.004) for the GlcN-HCl pre-treatment samples, when compared to the placebo pre-treatment samples (Fig. 4, panel B and C).

Sampling size calculations

The results of the sampling size calculations can be found in table 7. These sampling sizes are only valid when the same tissue processing and gene expression analysis procedures and protocols are used as we described in the materials and methods section of this study.

Table 7. Sampling size for testing cartilage gene expression *in vivo* based on placebo data of the GlcN-HCl and GlcN-S samples

Gene	Normalized gene expression	
	≥ 2-fold placebo (n per group)	**≤ 2-fold placebo (n per group)**
AGC1	4	17
COL2	3	13
MMP1	85	342
MMP2	32	128
MMP3	10	41
MMP9	49	195
MMP13	19	77
MMP14	10	41
ADAMTS4	21	85
ADAMTS5	13	51
TIMP1	12	50
TIMP2	6	23

DISCUSSION

One of the biggest concerns in all publications considering *in vitro* studies for the possible working mechanism of GlcN is the applicability to the *in vivo* situation. The concentrations used in culture experiments are very often much higher when compared to the concentrations measured in joint fluid after administration of GlcN at therapeutic dosage. Recently, Silbert found maximum serum GlcN levels in humans of 2-12 μM two to three hours after oral ingestion of 1500 mg GlcN-S after an overnight fast and Persiani et al. reported synovial GlcN concentration between 3 and 18 μM after oral administration of 1500 mg GlcN-S once daily on fourteen consecutive days in human subjects [26,27]. Most presented *in vitro* effects of GlcN on cartilage, including those from our recent publications, have been obtained using concentrations in the millimolar range [23-25]. One way to bypass this *in vitro* versus *in vivo* concentration issue, is to administer GlcN and a placebo control to patients and then assess the response of the cartilage in these patients. This way all bioavailability issues are bypassed and other factors concerning the artificial situation of culture experiments are eliminated also. To our knowledge, this is the first study that explores the effects of GlcN on the gene expression of cartilage and bone in patients with hip OA *in vivo*.

In this explorative *in vivo* pilot study we found indications of a down-regulation of aggrecan gene expression of hip cartilage after administration of GlcN-HCl.

With the outcome of the study by Rozendaal et al. being that there is no beneficial effect on pain or function of GlcN-S on patients with hip OA [32], and the fact that the only positive effects of GlcN-S over placebo published until now were in patients with knee OA, it would be interesting to explore whether a difference could be found between the *in vitro* data on gene expression after GlcN administration of hip and knee cartilage [2,4,5]. Whether direct translation of the results obtained with GlcN use in patients with knee OA to those with hip OA is valid, was

already questioned out by other authors [6]. Gene expression patterns in chondrocytes, isolated from osteoarthritic knee and hip joints were compared and found not to be different [33]. In one of our earlier publications using human osteoarthritic knee cartilage explants we found that gene expressions of aggrecan and collagen type II were significantly down-regulated after addition of 5 mM GlcN-HCl and 5 mM GlcN-S, when compared to control [23]. In this earlier study, no effect of the 0.5 mM concentration of both derivatives was found. In the present culture experiments using hip cartilage explants 0.5 mM GlcN-HCl seemed to have an effect. It appeared that this effect was most likely determined by the samples from patients who were pre-treated with GlcN-HCl before the samples were cultured, although differences in sensitivity of hip and knee cartilage for GlcN can not be excluded.

The number of studies investigating the effects of GlcN *in vitro*, using human osteoarthritic cartilage is still limited. It is not clear whether GlcN intake by the patients whose cartilage is used for culture experiments alters the outcome of these culture experiments. The gene expression data from the culture study in the GlcN-HCl study makes us concern whether that pre-treatment with GlcN alters the cell's responsiveness in subsequent *in vitro* studies. When we did not consider the specific pre-treatment (i.e., considering the GlcN-HCl and placebo pre-treatment groups as one group) we found indications for a down-regulation of the gene expression of aggrecan and collagen type II after culturing with 0.5 mM GlcN-HCl when compared to the control condition. When we did consider pre-treatment, we again found indications for a down-regulation of the gene expression of aggrecan and collagen type II but only in the GlcN-HCl pre-treatment group and not in the placebo pre-treatment group.

Furthermore, when comparing placebo and GlcN-HCl pre-treatment groups within the control and the 0.5 mM GlcN-HCl culture samples we again found indications for a lower gene expression of the GlcN-HCl pre-treated samples, considering aggrecan and collagen type II gene expression. For aggrecan this was found in both the control and 0.5 mM GlcN-HCl condition and for collagen type II only for the 0.5 mM GlcN-HCl condition. It thus seems that the use of GlcN-HCl *in vivo* may have primed the cartilage in such that its sensitivity to adding GlcN-HCl *in vitro* increased.

This pilot study was designed to explore whether it is possible to detect changes in gene expression after pre-operative oral GlcN administration and subsequent culture experiments. Although this is an explorative study and the expression of 12 different genes was assessed, it is remarkable that the same genes (aggrecan and collagen type II) that were earlier found to be down-regulated after addition of GlcN-HCl [23] again showed a trend towards down-regulation for GlcN-HCl. Thus, future sufficiently powered *in vivo* studies to replicate the results of this pilot study for these genes are needed. Considering the necessary sampling size to detect differences between placebo and GlcN for these genes we calculated a sampling size between 13 and 17 per group for such studies.

In conclusion, we believe that it is possible to detect changes in gene expression of cartilage after oral use of a supposed clinically effective daily dose of GlcN. Regarding the feasibility of

such studies, we provided in this study the genes of interest that can be investigated and a reasonable sampling size.

ACKNOWLEDGEMENTS

The authors would like to thank P. Pavljasevic, J. Koç and C. Feijt for their help with RNA isolation, M. Favejee and L. Jansen for their help with the logistics of the GlcN-HCl study, all the orthopaedic surgeons in the Rotterdam area who cooperated to provide the femoral heads of the patients in the GlcN-S study, and above all the patients who participated in both trials.

REFERENCES

1. Hathcock JN, Shao A. Risk assessment for glucosamine and chondroitin sulfate. Regul Toxicol Pharmacol 2007;47:78-83.
2. Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, *et al.* Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. N Engl J Med 2006;354:795-808.
3. Sawitzke AD, Shi H, Finco MF, Dunlop DD, Bingham CO, 3rd, Harris CL, *et al.* The effect of glucosamine and/or chondroitin sulfate on the progression of knee osteoarthritis: a report from the glucosamine/chondroitin arthritis intervention trial. Arthritis Rheum 2008;58:3183-91.
4. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, *et al.* Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-6.
5. Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002;162:2113-23.
6. Callaghan JJ, Buckwalter JA, Schenck RC, Jr. Argument against use of food additives for osteoarthritis of the hip. Clin Orthop 2000:88-90.
7. Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. Osteoarthritis Cartilage 1998;6:427-34.
8. Dodge GR, Jimenez SA. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis Cartilage 2003;11:424-32.
9. Bassleer C, Henrotin Y, Franchimont P. In-vitro evaluation of drugs proposed as chondroprotective agents. Int J Tissue React 1992;14:231-41.
10. Lippiello L. Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress. Osteoarthritis Cartilage 2003;11:335-42.
11. Vidal y Plana RR, Bizzarri D, Rovati AL. Articular cartilage pharmacology: I. In vitro studies on glucosamine and non steroidal antiinflammatory drugs. Pharmacol Res Commun 1978;10:557-69.
12. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:444-51.
13. Orth MW, Peters TL, Hawkins JN. Inhibition of articular cartilage degradation by glucosamine-HCl and chondroitin sulphate. Equine Vet J Suppl 2002:224-9.
14. de Mattei M, Pellati A, Pasello M, de Terlizzi F, Massari L, Gemmati D, *et al.* High doses of glucosamine-HCl have detrimental effects on bovine articular cartilage explants cultured in vitro. Osteoarthritis Cartilage 2002;10:816-25.
15. Anderson CC, Cook JL, Kreeger JM, Tomlinson JL, Wagner-Mann CC. In vitro effects of glucosamine and acetylsalicylate on canine chondrocytes in three-dimensional culture. Am J Vet Res 1999;60: 1546-51.
16. Ilic MZ, Martinac B, Handley CJ. Effects of long-term exposure to glucosamine and mannosamine on aggrecan degradation in articular cartilage. Osteoarthritis Cartilage 2003;11:613-22.
17. Gouze JN, Bianchi A, Becuwe P, Dauca M, Netter P, Magdalou J, *et al.* Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-kappa B pathway. FEBS Lett 2002;510:166-70.
18. Piperno M, Reboul P, Hellio Le Graverand MP, Peschard MJ, Annefeld M, Richard M, *et al.* Glucosamine sulfate modulates dysregulated activities of human osteoarthritic chondrocytes in vitro. Osteoarthritis Cartilage 2000;8:207-12.
19. Gouze JN, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, *et al.* Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis:

influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. Arthritis Rheum 2001;44:351-60.
20. Fenton JI, Chlebek-Brown KA, Caron JP, Orth MW. Effect of glucosamine on interleukin-1-conditioned articular cartilage. Equine Vet J Suppl 2002:219-23.
21. Byron CR, Orth MW, Venta PJ, Lloyd JW, Caron JP. Influence of glucosamine on matrix metalloproteinase expression and activity in lipopolysaccharide-stimulated equine chondrocytes. Am J Vet Res 2003;64:666-71.
22. Mello DM, Nielsen BD, Peters TL, Caron JP, Orth MW. Comparison of inhibitory effects of glucosamine and mannosamine on bovine articular cartilage degradation in vitro. Am J Vet Res 2004;65:1440-5.
23. Uitterlinden EJ, Jahr H, Koevoet JL, Jenniskens YM, Bierma-Zeinstra SM, Degroot J, *et al.* Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. Osteoarthritis Cartilage 2006;14:250-7.
24. Uitterlinden EJ, Jahr H, Koevoet JL, Bierma-Zeinstra SM, Verhaar JA, Weinans H, *et al.* Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate. Osteoarthritis Cartilage 2007;15:1267-74.
25. Uitterlinden EJ, Koevoet JL, Verkoelen CF, Bierma-Zeinstra SM, Jahr H, Weinans H, *et al.* Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants. BMC Musculoskelet Disord 2008;9:120.
26. Silbert JE. Dietary glucosamine under question. Glycobiology 2009;19:564-7.
27. Persiani S, Rotini R, Trisolino G, Rovati LC, Locatelli M, Paganini D, *et al.* Synovial and plasma glucosamine concentrations in osteoarthritic patients following oral crystalline glucosamine sulphate at therapeutic dose. Osteoarthritis Cartilage 2007;15:764-72.
28. Svarstad BL, Chewning BA, Sleath BL, Claesson C. The brief medication questionnaire: a tool for screening patient adherence and barriers to adherence. Patient Educ Couns 1999;37:113-24.
29. Rozendaal RM, Koes BW, Weinans H, Uitterlinden EJ, van Osch GJ, Ginai AZ, *et al.* The effect of glucosamine sulphate on osteoarthritis: design of a long-term randomised clinical trial [ISRCTN54513166]. BMC Musculoskelet Disord 2005;6:20.
30. Mandl EW, Jahr H, Koevoet JL, van Leeuwen JP, Weinans H, Verhaar JA, *et al.* Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture. Matrix Biol 2004;23:231-41.
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.
32. Rozendaal RM, Koes BW, van Osch GJ, Uitterlinden EJ, Garling EH, Willemsen SP, *et al.* Effect of glucosamine sulfate on hip osteoarthritis: a randomized trial. Ann Intern Med 2008;148:268-77.
33. Hoberg M, Aicher WK, Wulker N, Rudert M. [Expression analysis of different collagens and cytokines in cartilage cells derived from arthrotic hip and knee joints] Expressionsanalyse verschiedener Kollagene und Zytokine in Knorpelzellen aus arthrotisch veranderten Huft- und Kniegelenken. Z Orthop Ihre Grenzgeb 2006;144:289-95.

Chapter 7

Summary & General Discussion

With the ongoing aging of the population worldwide, osteoarthritis (OA) is a disease that will affect increasing numbers of people leading to an increasing personal, as well as socio-economic, burden. Until now, the only non-operative measures available to treat the OA-related symptoms were, for example, pain medication and physical therapy. If these conservative measures prove to be no longer sufficient, the only remaining possibility to restore joint function and relieve pain is joint replacement surgery.

In the last decades, the so-called 'disease-modifying osteoarthritis drugs' (DMOAD) have received considerable interest. Glucosamine (GlcN) is one of the compounds which is claimed to slow down OA progression and to have a beneficial effect on OA-related symptoms when compared to placebo treatment [1-5]. Therefore, manufacturers of this food additive have claimed that GlcN is a new treatment modality in OA with actual disease-modifying properties. Since it is a food additive, manufacturers are not required to meet the strict regulations regarding safety and effectiveness that apply to regular pharmaceuticals.

The task of investigating these additives can best be performed by independent research institutions, with the aim to confirm or refute the claims of the commercial manufacturers. The main goal of this thesis was to further elucidate the working mechanism of GlcN in OA, with the aim to offer well-founded recommendations regarding its use in patients suffering from OA.

GLUCOSAMINE IN RELATION TO DIFFERENT METABOLIC STATES OF CARTILAGE

It is important to realize that the effects of GlcN are influenced by differences in metabolic circumstances of cartilage. In the study presented in chapter 3 we used bovine chondrocytes seeded in alginate beads. A balance between extracellular matrix (ECM) synthesis and degradation was created *in vitro* by a prolonged pre-culture period of 3 weeks to allow the chondrocytes to create an ECM within the alginate beads. Hereafter the beads were treated for 14 days with the experimental conditions. In this situation, no effect of glucosamine-hydrochloride (GlcN-HCl) or glucosamine-sulphate (GlcN-S) addition was found on the amount of glycosaminoglycan (GAG). Since in most healthy people ECM degradation and synthesis in cartilage is in balance, these data suggest that no beneficial effect of the use of this food additive can be expected in this situation. In other words, if nothing is wrong with a person's cartilage, preventive use of GlcN (which can, for instance, be found in sports drinks) is not necessary.

When an ECM still has to be formed in the alginate beads, the seeded chondrocytes are believed to be in a mainly anabolic environment. *In vitro* this was created by adding the experimental conditions for 14 days directly following the 3-day pre-culture period, since in this situation no ECM has yet been formed. Here we found that addition of 0.5 and 5 mM GlcN-S or GlcN-HCl led to significantly less GAG per bead when compared to the control. This reduced GAG amount was in accordance with the down-regulated expression of genes involved in

anabolic activities in human osteoarthritic cartilage explants by GlcN (described in chapter 2). Thus, based on these results, rebuilding or restoration of cartilage is not to be expected. In the early 1980s, Mankin et al. showed that osteoarthritic chondrocytes are metabolically hyperactive in order to repair cartilage damage, at least early in the process [6]. From this viewpoint it is questionable whether the use of GlcN in the earlier OA stages is advisable, since our results suggest that GlcN might interfere with this natural repair process in OA.

In chapter 3 the catabolic situation was created by addition of interleukin-1β for 48 hours to the cultures with bovine chondrocytes seeded in alginate beads, and in chapter 2 through the use of human osteoarthritic cartilage explants. In these catabolic situations we found some effects of GlcN that might be beneficial for patients with OA. In the bovine chondrocytes it was found that GlcN pre-treatment protected against the interleukin-1β mediated reduction in total GAG per bead. In the human explant study, addition of GlcN down-regulated several genes involved in the catabolic processes. A lower total matrix metalloproteinases (MMP) activity, when compared to the control, was found as well. Thus, in a mainly catabolic environment, GlcN seems to protect cartilage from several ECM degrading activities, and therefore might be useful in OA.

In conclusion, if a healthcare worker were to advise GlcN use to a patient suffering from OA, this should only be based on the possible counteraction of further cartilage degradation and not with the aim to restore already damaged cartilage.

DIFFERENCES BETWEEN GLUCOSAMINE DERIVATIVES

The three GlcN derivatives most frequently investigated in the literature, namely GlcN-HCl, GlcN-S and N-acetyl-glucosamine (GlcN-Ac), were also used in this thesis. The question is whether or not these derivatives have the same effects in cartilage. When comparing GlcN-S and GlcN-HCl on the one hand, and GlcN-Ac on the other, we found remarkable opposite effects on cartilage. In the alginate model in chapter 3, GlcN-Ac showed an increase in the amount of GAG per bead in the situation with a balance between ECM degradation and synthesis, whereas the other two compounds did not. In the same study, in the situation where the ECM still had to be formed (i.e., anabolic environment) no effect of GlcN-Ac was found, as opposed to a reduced amount of GAG per bead after addition of GlcN-S or GlcN-HCl. Similar opposite results of GlcN-Ac compared to other forms of GlcN were also found by Terry et al. [7]. Recently, Shikhman et al. also showed that GlcN and GlcN-Ac differ in uptake, effects on glucose transport, glucose transporter expression, GAG and hyaluronic acid (HA) synthesis, indicating that they have distinct molecular mechanisms mediating their differential biological activities in chondrocytes [8]. In the *in vitro* studies presented in this thesis it was shown that the effects of GlcN depend on the concentration of glucose that is present. In joint effusions of knees with OA, mean glucose concentration was 5.4 mM, with a range comparable to the reference range for serum [9]. Therefore, we used a medium

with a low glucose concentration (1000 mg/l = 5.55 mM) in all culture experiments, since this best mimics the physiological situation. Mroz and Silbert showed that the ratio of glucose to GlcN concentration *in vitro* is important for the utilization of GlcN [10,11]. Their results suggest that not only the absolute concentration of GlcN, but also the GlcN to Gluc ratio plays an important role in the utilization and therefore effectiveness of exogenously provided GlcN. In cultured mouse chondrocytes and immortalized human chondrocytes they found that exogenous GlcN was incorporated in newly formed chondroitin sulphate when added at an equimolar concentration with glucose in the culture medium. When glucose concentration became higher than the GlcN concentration, cells utilized less exogenous GlcN for the formation of chondroitin sulphate, but preferably incorporated GlcN that was endogenously formed from glucose. With this equimolar ratio in mind, in our experiments we decided to add GlcN to the medium at concentrations equimolar to (or lower than) that of medium glucose. In chapter 3, further indications were found that glucose can interfere with the effects of GlcN. Here, higher medium glucose concentrations prevented GlcN from inhibiting GAG production, as also reported earlier [12]. This pattern was more pronounced for GlcN-HCl than for GlcN-Ac. With medium Gluc concentration of 15 or 25 mM the significantly reduced GAG content per bead, due to GlcN-HCl addition, was no longer present. Based on these findings and the results from others, we hypothesized that GlcN-HCl or GlcN-S addition might lead to intracellular adenosine triphosphate (ATP) depletion due to competition between these compounds and glucose at different cellular levels [13-21]. This depletion of ATP, the universal energy source in cellular metabolism, results in less energy for all intracellular processes like ECM production and breakdown. A recent report on vascular smooth muscle cells, confirmed this depletion of cellular ATP content due to GlcN [22]. The reason why GlcN-Ac does not inhibit matrix synthesis may be because it is phosphorylated by a different enzyme and thus no competition with Gluc occurs, while the phosphorylated GlcN-Ac can also be used for GAG synthesis [23].

Our results, combined with the data from others, lead to the conclusion that GlcN-Ac should be regarded as a different entity when compared to GlcN-S and GlcN-HCl. The question then remains: can a difference be found between GlcN-S and GlcN-HCl, or are their effects similar? It has been hypothesized that sulphate, and not GlcN itself, mediates the clinical effects of GlcN-S and, therefore, that GlcN salts other than GlcN-S would be ineffective [24]. In the human osteoarthritic knee explant study (chapter 2) similar effects of GlcN-S and GlcN-HCl were found on gene expression. For example, a significant down-regulation was found for ADAMTS4 (a desintegrin and a metalloproteinase with trombospondin motifs) gene expression, i.e., aggrecanase-1, for the 5 mM concentration of both GlcN derivatives. Considering the MMPs, a significant down-regulation was found for MMP3 for both the 5 mM GlcN-HCl and GlcN-S derivative. Furthermore, addition of the 5 mM concentration of GlcN-HCl and GlcN-S led to a significant down-regulation of both aggrecan and collagen type II in the human osteoarthritic explants, when compared to controls. Median gene expression of ADAMTS5, i.e., aggrecanase-2, and TIMP3 (the natural tissue inhibitors of MMPs) were down-regulated by both derivatives, but only

significantly for 5 mM GlcN-S. Directly comparing the effects of treatment with 5 mM GlcN-HCl and 5 mM GlcN-S showed that expression of ADAMTS5, TIMP3 and aggrecan was significantly more down-regulated by 5 mM GlcN-S. The data in chapter 3 showed similar effects after addition of GlcN-S or GlcN-HCl. Thus, based on the results of our *in vitro* experiments, we believe that the effects mediated by GlcN are relatively independent from whether the sulphate or the hydrochloride salt is used.

When considering the effects of GlcN in patients, the discussion on equal effectiveness of GlcN-S and GlcN-HCl continues [25]. As Houpt et al. stated earlier, GlcN salts (hydrochloride and sulphate) are fully ionized in the acid environment of the stomach, making GlcN free for absorption in the small intestine [5]. Furthermore, Deal et al. reported that GlcN-HCl has a higher compound purity than GlcN-S (99% vs 74%) [26]. A study on the heterogeneity of GlcN trials concluded that GlcN-HCl is not effective [27]. The updated Cochrane review by Towheed et al. in 2005, showed that only the GlcN-S preparation of the Rotta company was superior to placebo in the OA-related functional impairment and pain and that other, non-Rotta, GlcN preparations were not effective [25]. In chapter 5 we also found no effects of GlcN-S in subgroups of patients with hip OA. This was a subgroup analysis of a randomized controlled trial performed at our institution which included 222 primary care patients. We investigated whether an effect of GlcN-S treatment could be found on symptoms and structural progression of hip OA over a 2-year period. Details of the study protocol of this trial can be found in the Appendix of this thesis. In chapter 6, patients from the outpatient clinic of the Erasmus MC Rotterdam received GlcN-HCl or placebo 8-10 weeks prior to scheduled total hip replacement surgery (GlcN-HCl study). In this explorative study we also reported on the effects of GlcN-HCl on the Western Ontario McMaster Universities (WOMAC) and visual analogue scale pain scores. However, sampling size in this GlcN-HCl study was too small to draw statistically valid conclusions from the pain related data.

Furthermore, in chapter 6 we explored whether changes in gene expression of femoral head cartilage could be detected in patients with hip OA after pre-operative oral GlcN use. This was analysed for the patients in the aforementioned GlcN-HCl study and for a second group of patients, namely those who underwent total hip replacement surgery during the 2-year course of the trial described in chapter 5 (GlcN-S study). When exploring the gene expression data in the GlcN-HCl study we found indications of a down-regulation of aggrecan gene expression *in vivo* for GlcN-HCl when compared to placebo (normalised expression 1.40 ± 0.97 vs 3.03 ± 1.06). Exploration of the gene expression data in the GlcN-S study in chapter 6 did not reveal any indication of significantly altered gene expression of cartilage *in vivo*. A possible reason why we found indications of a difference in gene expression *in vivo* in the GlcN-HCl study, but not in the GlcN-S study, might be because the duration of study medication was controlled in the GlcN-HCl study but not in the GlcN-S study. Furthermore, a very different placebo to compound ratio existed between the two studies. However, since the study in chapter 6 was intended as an

explorative pilot study, the indications of down-regulation we observed should be replicated in future studies. Directions for sampling size were given in chapter 6 as well.

In this thesis we found similar effects of GlcN-HCl and GlcN-S *in vitro*, but *in vivo* indications were found that GlcN-HCl and not GlcN-S affected cartilage gene expression. Therefore, unfortunately, the results emerging from this thesis do not fully resolve the ongoing discussion regarding the equal effectiveness of GlcN-S and GlcN-HCl in patients with OA.

EFFECTS OF GLUCOSAMINE ON TISSUES OTHER THAN CARTILAGE

OA is a disease that affects all components of the joint and not only the articular cartilage. The lay term 'cartilage wear', frequently used to indicate OA, is factually incorrect since the homoeostasis of the entire joint is disturbed in the presence of OA. Thus, apart from the effects of GlcN on cartilage described above, we also investigated the effects on synovium (chapter 4) and in chapter 6 the effects on bone were explored. While exploring the bone gene expression data, we only observed a down-regulation of MMP13 for GlcN-S when compared to placebo (normalised expression 0.11 ± 0.14 vs 0.57 ± 0.33; $p = 0.048$) at the top layer of trabecular subchondral bone. For all the other levels and genes tested, no significant differences were observed. However, due to technical difficulties during tissue processing and RNA extraction a fair number of data was missing. Thus, for valid conclusions considering the effects of GLcN on bone, future studies are needed to confirm or deny our observation.

In chapter 4, addition of 0.5 and 5 mM GlcN-HCl to human osteoarthritic synovium explants *in vitro* led to an approximately 2-4 fold higher amount of medium HA concentration when compared to the non-supplemented control, without affecting hyaluronic acid synthase gene expression. For GlcN-Ac no effect on medium HA or hyaluronic acid synthase gene expression was found. It was earlier suggested that GlcN might exert its effects in OA by influencing HA synthesis, since it is a rate-limiting precursor in HA synthesis [28,29]. The findings in chapter 4 show that GlcN does influence HA synthesis in human OA synovium *in vitro*. The increased HA concentration might be important in influencing OA, since HA plays a major role in joint lubrication and maintaining joint homeostasis [30]. HA was also shown to have anti-inflammatory effects on OA-specific cytokines by down-regulation of tumor necrosis factor α, interleukin-8 and inducible nitric oxide synthase [31]. Since cartilage does not contain nerve endings, the pain relief experienced by OA patients after GlcN use cannot be explained by effects on cartilage. We then suggested that the increased HA concentration due to GlcN might explain the alleged effects on pain in OA, while viscosupplementation with HA in knee OA was earlier shown to improve pain and function [32]. However, the size and concentration of HA play an important role in the viscoelastic properties, and the regulation of inflammatory and tissue repair responses [33,34]. The concentration and molecular weight of HA are reported to be lower in inflammatory joints compared to healthy joints [35]. By adding GlcN we found an increase in HA concentration, which

might be beneficial. However the assay we used only measured total HA concentration and not the molecular weight of the HA molecules. Thus, we do not know whether the molecular weight of the extra HA was high enough to restore the viscoelastic properties and be beneficial in OA. This could be a subject for future investigation.

GLUCOSAMINE AND HIP VERSUS KNEE OA

Most evidence on the positive clinical effects of GlcN is based on patients with knee OA [25]. Whether or not these results are also applicable to hip OA has been questioned earlier [36]. In relation to this issue, pain and function scores were reported for patients with hip OA in chapter 5 and 6 of this thesis. In chapter 5, additional analyses examined subgroups based on baseline joint space width, concomitant knee OA at baseline, pain level, and use of pain medication. Overall, no significant differences were found between GlcN-S and placebo. The exploratory subgroup analysis of patients with concomitant knee OA showed an adjusted difference for WOMAC pain of -5.7 (95% confidence interval: -12.6, 1.3), in favor of GlcN-S. In patients who did not have radiographic knee OA at baseline this difference was -0.1 (95% confidence interval -4.9, 4.7). An explanation for this non-significant difference within this particular subgroup might be that GlcN-S had an effect on the knee OA symptoms such that patients experienced less pain as was registered with the questionnaires for hip complaints. However, since this difference is not significant, the question whether or not GlcN has different effects on symptoms in hip or knee OA (or the combination of both), needs to be further elucidated.

Pending the results of such a study, we offer a preliminary explanation for a possible difference in effects of GlcN on knee and hip OA. With regards to joint anatomy, the hip and knee joints have a different kind of articulation. Firstly, with its ball-and-socket design the hip has much more intrinsic stability than the knee joint. To overcome this relative instability, the knee joint is supported by the menisci, and the collateral and cruciate ligament complex. In the course of OA the menisci also suffer from degenerative changes that can themselves lead to complaints in patients. Furthermore, ligament laxity also plays a role in the development of knee OA [37]. This causes different load distribution patterns within the knee joint, leading to more localized than generalized pain within that joint. These specific mechanisms of the knee joint are less abundant in the hip, implying that the knee joint has more aspects that might benefit from intervention. Secondly, the hip joint consists of one articulation (the femoro-acetabular joint), whereas the knee consists of two (the femoro-tibial and patello-femoral joints). Taking this into account, the knee has more locations from which pain can originate and, therefore, more possible locations where GlcN can exert its effects.

Since cartilage is not innervated, the pain reduction described for knee OA after the use of GlcN can not be explained by direct effects on cartilage tissue. One explanation for the effects of GlcN on pain in knee OA is that GlcN interferes with the inflammatory process that occurs

in OA. In patients with knee OA, moderate and large effusions and synovial thickening were more frequent among patients with knee pain than in those without [38]. This suggests that these features are associated with the pain of knee OA. Thus, interference of GlcN with this inflammatory process might lead to pain reduction in knee OA. Also, because joint volume and the synovial lining area in the hip is less than in the knee, the inflammatory 'load' in the hip may be less compared to the knee. Thus, the effect of GlcN on the inflammatory process (and thereby the reduction of pain in hip OA) might be less in the hip than in knee OA. Another indication that hip OA might differ from knee OA is that both joints react differently to interventional therapies involving joint lubrication and homeostasis. Intra-articular supplementation with HA showed beneficial effects on pain and function in patients with knee OA [32]. In contrast, Qvistgaard et al. found no significant effect of intra-articular HA injection in patients with hip OA [39]. In chapter 4 we stated that the increased amount of HA after addition of GlcN to knee synovium explants could be responsible for the alleged effects of GlcN on pain. With regard to the study of Qvistgaard et al., this might not automatically be valid for GlcN and hip synovium as well, although this should be confirmed in future experiments.

Since the only positive effects of GlcN over placebo were found in patients with knee OA, and no beneficial effect of GlcN was found in patients with hip OA (chapter 5), we decided to investigate whether there is a difference in the gene expression data of hip and knee cartilage after GlcN administration [1,2,40]. Gene expression patterns in chondrocytes (isolated from osteoarthritic knee and hip joints) have been compared and were found not to be different [41]. Unfortunately, because *in vivo* gene expression data were only available for hip cartilage (chapter 6) and not for knee cartilage, a direct knee versus hip comparison can only be performed based on the *in vitro* data from the explant culture studies in chapter 2 (knee) and chapter 6 (hip).

We compared the gene expression data of aggrecan and collagen type II, since these two genes were the only ones for which a significant effect was observed in both the *in vitro* studies in chapter 2 and 6. We compared the Ct values of these two genes of interest normalized to glyceraldehyde 3-phosphate dehydrogenase, for the control and 0.5 mM GlcN-HCl conditions. A significant difference was found in gene expression of aggrecan normalized to glyceraldehyde 3-phosphate dehydrogenase when comparing the control conditions of the knee and hip cartilage explants. Since a difference was found between the control conditions of both studies, we decided that a comparison of the experimental 0.5 mM GlcN-HCl condition would no longer be valid. For collagen type II the gene expression of the control conditions were not significantly different, but neither were the collagen type II gene expression data of the 0.5 GlcN-HCl condition of both studies. This implies that there is no difference in the effect of GlcN on hip or knee cartilage. However, since the explant material used for the *in vitro* experiments (chapters 2 and 6) might not be fully comparable, this (indirectly obtained) conclusion should be interpreted with caution. It would be interesting to directly compare the effect of GlcN on gene expression in hip and knee cartilage explants in a well-controlled *in vitro* study, with comparable baseline material.

STUDY LIMITATIONS: *IN VITRO* VERSUS *IN VIVO*; EXPERIMENTAL GLUCOSAMINE CONCENTRATIONS

At the time we designed the trials described in this thesis, the GlcN concentrations used in many earlier reports on *in vitro* studies with GlcN and cartilage or chondrocytes were much higher than the concentrations reported nowadays in human synovium [12,42-50]. Based on the formerly accepted GlcN concentrations for *in vitro* studies, and bearing in mind the importance of the glucose to GlcN ratio as presented by Mroz and Silbert, we chose the GlcN concentrations for our *in vitro* experiments [10,11]. We continued to use those concentrations in all our subsequent studies in order to maintain inter-study comparability.

Silbert showed maximum GlcN serum levels in humans of 2-12 µM 2-3 hours after ingestion of 1500 mg GlcN-S after an overnight fast [51]. Persiani et al. demonstrated that synovial GlcN concentration after oral administration at the therapeutic dose of 1500 mg once-a-day, varied between 3 and 18 µM [50]. These concentrations are much lower than the concentrations we used in the *in vitro* experiments in this thesis. Thus, although our choice of GlcN concentrations for the *in vitro* experiments was well considered, this now turns out to be a major limitation regarding the clinical relevance of our *in vitro* results. On the other hand, it can be questioned whether the GlcN concentration directly surrounding the chondrocytes is in fact as low as reported by Persiani et al. In a study using GlcN-HCl administered to horses it was found that although synovial fluid GlcN concentrations were less than 10% of the serum concentrations obtained at the same time, GlcN was still detectable in synovial fluids 6 hours after it was nearly completely cleared from the serum [52]. Other studies that administered radioactively labelled GlcN to animals also reported that articular cartilage has in some way the capacity to retain and accumulate GlcN [53-55]. This was also shown in six healthy male volunteers using a single dose ^{14}C labelled GlcN-S orally, intravenous or intramuscular [56]. All these studies indicate that GlcN is not only retained within the joint, but is also accumulated in articular cartilage. Therefore, concentrations of GlcN within the articular cartilage can actually be much higher than those found in the surrounding synovial fluid. In a recent report on horses, it was shown that mean synovial fluid GlcN levels were more than four-fold higher in inflamed joints when compared to healthy joints [57]. Therefore, in OA patients synovial fluid GlcN concentrations might be even higher than in healthy volunteers.

In relation to the importance of the GlcN to glucose ratio, the study of Windhaber et al. [14] is worth mentioning. Based on measurements with microelectrodes, they reported that glucose concentration directly surrounding the chondrocytes was 1 mM. Based on this finding, and bearing in mind the importance of the GlcN to glucose ratio, to exert an effect *in vivo* that is similar to that found in our *in vitro* experiments, the GlcN concentrations may not need to be as high as the concentrations we used *in vitro*. Indications for this were observed in chapter 6 where the *in vitro* versus *in vivo* concentration issue was bypassed by administering GlcN (1500 mg once daily) or placebo to patients and then assessing what happened to cartilage

gene expression in these patients. In this explorative pilot study we found indications for down-regulation of aggrecan gene expression of hip cartilage after administration of GlcN-HCl when compared to placebo. This corresponds with our findings reported in chapter 2 for human osteoarthritic knee cartilage explants *in vitro*. Moreover, in the *in vitro* experiments in chapter 6 we found indications that GlcN might react different to GlcN in culture when it was pretreated with GlcN *in vivo*, when compared to pretreatment with placebo *in vivo*.

IMPLICATIONS FOR FUTURE RESEARCH

Based on the results in this thesis, GlcN is not expected to be able to restore damaged cartilage or to play a role in maintaining normal healthy cartilage. Although we hypothesized that GlcN might protect cartilage against enzymatic ECM breakdown, this idea was based on short-term *in vitro* studies. This also needs to be proven in well-designed long-term studies. Since long-term *in vitro* studies are not reliable this should be investigated *in vivo* or by using animal models. However, the alleged structure-modifying properties on cartilage still do not explain the effects on pain that were reported in earlier studies after ingestion of GlcN [58]. Based on our findings in chapter 4, we hypothesized that the increase of HA after adding GlcN *in vitro* might be the basis of the reported effects of GlcN on pain reduction. In our opinion, the increase of HA after addition of GlcN to synovium we found in chapter 4 is the most promising finding for the future use of GlcN. If there is additional research on GlcN, we suggest to investigate whether the extra HA that is formed has the appropriate molecular weight. Compared to the synovial GlcN concentrations reported in humans after oral ingestion we used relatively high GlcN concentrations *in vitro*. In future experiments lower GlcN concentrations should be used *in vitro* or *in vivo*, as we did in chapter 6 in order to bypass the concentration issue.

During the past decade, that started with promising reports of GlcN acting as a DMOAD, doubts have increased about its clinical efficacy [1,2,59-61]. Therefore the search for DMOADs, other than GlcN, will continue. Some examples of compounds with alleged disease-modifying properties in OA currently under investigation are: diacerein (an inhibitor of interleukin-1β), doxycycline (which acts as an MMP inhibitor), licofelone (a dual cyclo-oxygenase/5-lipoxygenase inhibitor), calcitonine, and blockers of inducible nitric oxide synthase (naproxcinod) [62,63]. Based on our results, and taking into account the limitations of the studies in this thesis, future investigations on the efficacy of promising DMOADs should consider the following aspects:

1) For *in vitro* studies the effects of the compound of interest should be tested on all the basic tissue types within a diarthrodial joint, i.e., bone, cartilage and synovium.
2) When designing an *in vitro* experiment the concentration of the compound under study should be as close as possible to that in (human) synovial fluid, given that such data exist at the moment the experiment is designed.

3) In experiments using chondrocytes in a 3-D environment, the status of the surrounding ECM can influence the outcome. Therefore, it must be established whether the chondrocytes are in a mainly anabolic state, or in a situation of balance between ECM synthesis and degradation.
4) If a model for OA is used, to create a mainly catabolic situation, the use of human osteoarthritic tissue (cartilage, synovium) obviously comes closest to the *in vivo* situation; we have shown that it is possible to use human tissue for testing the effects of a DMOAD.
5) When selecting patients whose material will be used in such experiments, pre-treatment with over-the-counter substances that may have disease-modifying properties should be considered, and the material of these patients should perhaps be excluded from *in vitro* experiments.
6) To bypass the translational conflict of the *in vitro* situation to clinical efficacy, the effects of DMOADs can also be investigated in patients. However, when designing this type of study it is important to be informed about the use of similar (non-prescription) disease-modifying compounds and it may be wise to exclude these patients from the trial.
7) When studying the effect of a DMOAD in patients, a method to obtain as much material for analysis as possible is to use the waste material from total joint replacement surgery. However, to deal with this the patient needs to use the DMOAD pre-operatively in a placebo-controlled trial. To increase the comparability of results, it is important that the duration of use of the active compound or placebo is as standardized as possible.
8) As an outcome parameter gene expression can be valuable, but complementary data on the change in the final products that these genes encode for should also be provided.

In conclusion, based on the findings in this thesis we believe that GlcN can not be expected to restore damaged cartilage. In OA, GlcN might protect cartilage against enzymatic ECM breakdown. Furthermore, GlcN might improve joint lubrication and homeostasis by increasing HA in synovial fluid. The major limitation of our *in vitro* experimental studies is that supra-physiological concentrations of GlcN were used. We hope that the lessons learned in our studies on GlcN, and future research on other DMOADs, will lead to the development of an effective treatment for OA in the future.

REFERENCES

1. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, *et al.* Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-6.
2. Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002;162:2113-23.
3. Herrero-Beaumont G, Ivorra JA, Del Carmen Trabado M, Blanco FJ, Benito P, Martin-Mola E, *et al.* Glucosamine sulfate in the treatment of knee osteoarthritis symptoms: a randomized, double-blind, placebo-controlled study using acetaminophen as a side comparator. Arthritis Rheum 2007;56:555-67.
4. Braham R, Dawson B, Goodman C. The effect of glucosamine supplementation on people experiencing regular knee pain. Br J Sports Med 2003;37:45-9; discussion 9.
5. Houpt JB, McMillan R, Wein C, Paget-Dellio SD. Effect of glucosamine hydrochloride in the treatment of pain of osteoarthritis of the knee. J Rheumatol 1999;26:2423-30.
6. Mankin HJ, Johnson ME, Lippiello L. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. III. Distribution and metabolism of amino sugar-containing macromolecules. J Bone Joint Surg Am 1981;63:131-9.
7. Terry DE, Rees-Milton K, Smith P, Carran J, Pezeshki P, Woods C, *et al.* N-acylation of glucosamine modulates chondrocyte growth, proteoglycan synthesis, and gene expression. J Rheumatol 2005;32: 1775-86.
8. Shikhman AR, Brinson DC, Valbracht J, Lotz MK. Differential metabolic effects of glucosamine and N-acetylglucosamine in human articular chondrocytes. Osteoarthritis Cartilage 2009;17:1022-8.
9. Krachler M, Domej W. Clinical laboratory parameters in osteoarthritic knee-joint effusions correlated to trace element concentrations. Biol Trace Elem Res 2001;79:139-48.
10. Mroz PJ, Silbert JE. Effects of [3H]glucosamine concentration on [3H]chondroitin sulphate formation by cultured chondrocytes. Biochem J 2003;376:511-5.
11. Mroz PJ, Silbert JE. Use of (3)H-glucosamine and (35)S-sulfate with cultured human chondrocytes to determine the effect of glucosamine concentration on formation of chondroitin sulfate. Arthritis Rheum 2004;50:3574-9.
12. Sandy JD, Gamett D, Thompson V, Verscharen C. Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. Biochem J 1998;335 (Pt 1):59-66.
13. Uldry M, Ibberson M, Hosokawa M, Thorens B. GLUT2 is a high affinity glucosamine transporter. FEBS Lett 2002;524:199-203.
14. Windhaber RA, Wilkins RJ, Meredith D. Functional characterisation of glucose transport in bovine articular chondrocytes. Pflugers Arch 2003;446:572-7.
15. Van Schaftigen E. Glucosamine-sensitive and -insensitive detritiation of [2-3H]glucose in isolated rat hepatocytes: a study of the contributions of glucokinase and glucose-6-phosphatase. Biochem J 1995;308 (Pt 1):23-9.
16. Virkamaki A, Yki-Jarvinen H. Allosteric regulation of glycogen synthase and hexokinase by glucosamine-6-phosphate during glucosamine-induced insulin resistance in skeletal muscle and heart. Diabetes 1999;48:1101-7.
17. Otte P. Basic cell metabolism of articular cartilage. Manometric studies. Z Rheumatol 1991;50:304-12.
18. Tesoriere G, Vento R, Calvaruso G. Inhibitory effect of D-glucosamine on glycolysis in bovine retina. Biochim Biophys Acta 1975;385:58-67.
19. Hresko RC, Heimberg H, Chi MM, Mueckler M. Glucosamine-induced insulin resistance in 3T3-L1 adipocytes is caused by depletion of intracellular ATP. J Biol Chem 1998;273:20658-68.

20. Lee RB, Urban JP. Evidence for a negative Pasteur effect in articular cartilage. Biochem J 1997;321 (Pt 1):95-102.
21. Baker MS, Feigan J, Lowther DA. The mechanism of chondrocyte hydrogen peroxide damage. Depletion of intracellular ATP due to suppression of glycolysis caused by oxidation of glyceraldehyde-3-phosphate dehydrogenase. J Rheumatol 1989;16:7-14.
22. Little PJ, Drennon KD, Tannock LR. Glucosamine inhibits the synthesis of glycosaminoglycan chains on vascular smooth muscle cell proteoglycans by depletion of ATP. Arch Physiol Biochem 2008;114: 120-6.
23. Hinderlich S, Berger M, Schwarzkopf M, Effertz K, Reutter W. Molecular cloning and characterization of murine and human N-acetylglucosamine kinase. Eur J Biochem 2000;267:3301-8.
24. Hoffer LJ, Kaplan LN, Hamadeh MJ, Grigoriu AC, Baron M. Sulfate could mediate the therapeutic effect of glucosamine sulfate. Metabolism 2001;50:767-70.
25. Towheed T, Maxwell L, Anastassiades T, Shea B, Houpt J, Robinson V, *et al.* Glucosamine therapy for treating osteoarthritis. Cochrane Database Syst Rev 2005:CD002946.
26. Deal CL, Moskowitz RW. Nutraceuticals as therapeutic agents in osteoarthritis. The role of glucosamine, chondroitin sulfate, and collagen hydrolysate. Rheum Dis Clin North Am 1999;25:379-95.
27. Vlad SC, LaValley MP, McAlindon TE, Felson DT. Glucosamine for pain in osteoarthritis: why do trial results differ? Arthritis Rheum 2007;56:2267-77.
28. McCarty MF. Enhanced synovial production of hyaluronic acid may explain rapid clinical response to high-dose glucosamine in osteoarthritis. Med Hypotheses 1998;50:507-10.
29. McCarty MF, Russell AL, Seed MP. Sulfated glycosaminoglycans and glucosamine may synergize in promoting synovial hyaluronic acid synthesis. Med Hypotheses 2000;54:798-802.
30. Laurent TC, Laurent UB, Fraser JR. The structure and function of hyaluronan: An overview. Immunol Cell Biol 1996;74:A1-7.
31. Wang CT, Lin YT, Chiang BL, Lin YH, Hou SM. High molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis. Osteoarthritis Cartilage 2006;14:1237-47.
32. Bellamy N, Campbell J, Robinson V, Gee T, Bourne R, Wells G. Viscosupplementation for the treatment of osteoarthritis of the knee. Cochrane Database Syst Rev 2006:CD005321.
33. Fam H, Bryant JT, Kontopoulou M. Rheological properties of synovial fluids. Biorheology 2007;44: 59-74.
34. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 2007;23: 435-61.
35. Dahl LB, Dahl IM, Engstrom-Laurent A, Granath K. Concentration and molecular weight of sodium hyaluronate in synovial fluid from patients with rheumatoid arthritis and other arthropathies. Ann Rheum Dis 1985;44:817-22.
36. Callaghan JJ, Buckwalter JA, Schenck RC, Jr. Argument against use of food additives for osteoarthritis of the hip. Clin Orthop 2000:88-90.
37. Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, *et al.* Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 2000;133:635-46.
38. Hill CL, Gale DG, Chaisson CE, Skinner K, Kazis L, Gale ME, *et al.* Knee effusions, popliteal cysts, and synovial thickening: association with knee pain in osteoarthritis. J Rheumatol 2001;28:1330-7.
39. Qvistgaard E, Christensen R, Torp-Pedersen S, Bliddal H. Intra-articular treatment of hip osteoarthritis: a randomized trial of hyaluronic acid, corticosteroid, and isotonic saline. Osteoarthritis Cartilage 2006;14:163-70.
40. Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, *et al.* Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. N Engl J Med 2006;354:795-808.
41. Hoberg M, Aicher WK, Wulker N, Rudert M. [Expression analysis of different collagens and cytokines in cartilage cells derived from arthrotic hip and knee joints] Expressionsanalyse verschiedener Kol-

lagene und Zytokine in Knorpelzellen aus arthrotisch veranderten Huft- und Kniegelenken. Z Orthop Ihre Grenzgeb 2006;144:289-95.
42. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:444-51.
43. de Mattei M, Pellati A, Pasello M, de Terlizzi F, Massari L, Gemmati D, *et al.* High doses of glucosamine-HCl have detrimental effects on bovine articular cartilage explants cultured in vitro. Osteoarthritis Cartilage 2002;10:816-25.
44. Fenton JI, Chlebek-Brown KA, Peters TL, Caron JP, Orth MW. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. Osteoarthritis Cartilage 2000;8:258-65.
45. Gouze JN, Bianchi A, Becuwe P, Dauca M, Netter P, Magdalou J, *et al.* Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-kappa B pathway. FEBS Lett 2002;510:166-70.
46. Gouze JN, Bordji K, Gulberti S, Terlain B, Netter P, Magdalou J, *et al.* Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis: influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes. Arthritis Rheum 2001;44:351-60.
47. Fenton JI, Chlebek-Brown KA, Caron JP, Orth MW. Effect of glucosamine on interleukin-1-conditioned articular cartilage. Equine Vet J Suppl 2002:219-23.
48. Mello DM, Nielsen BD, Peters TL, Caron JP, Orth MW. Comparison of inhibitory effects of glucosamine and mannosamine on bovine articular cartilage degradation in vitro. Am J Vet Res 2004;65:1440-5.
49. Byron CR, Orth MW, Venta PJ, Lloyd JW, Caron JP. Influence of glucosamine on matrix metalloproteinase expression and activity in lipopolysaccharide-stimulated equine chondrocytes. Am J Vet Res 2003;64:666-71.
50. Persiani S, Rotini R, Trisolino G, Rovati LC, Locatelli M, Paganini D, *et al.* Synovial and plasma glucosamine concentrations in osteoarthritic patients following oral crystalline glucosamine sulphate at therapeutic dose. Osteoarthritis Cartilage 2007;15:764-72.
51. Silbert JE. Dietary glucosamine under question. Glycobiology 2009;19:564-7.
52. Laverty S, Sandy JD, Celeste C, Vachon P, Marier JF, Plaas AH. Synovial fluid levels and serum pharmacokinetics in a large animal model following treatment with oral glucosamine at clinically relevant doses. Arthritis Rheum 2005;52:181-91.
53. Setnikar I, Giacchetti C, Zanolo G. Pharmacokinetics of glucosamine in the dog and in man. Arzneimittelforschung 1986;36:729-35.
54. Setnikar I, Giachetti C, Zanolo G. Absorption, distribution and excretion of radioactivity after a single intravenous or oral administration of [14C] glucosamine to the rat. Pharmatherapeutica 1984;3:538-50.
55. Setnikar I, Rovati LC. Absorption, distribution, metabolism and excretion of glucosamine sulfate. A review. Arzneimittelforschung 2001;51:699-725.
56. Setnikar I, Palumbo R, Canali S, Zanolo G. Pharmacokinetics of glucosamine in man. Arzneimittelforschung 1993;43:1109-13.
57. Meulyzer M, Vachon P, Beaudry F, Vinardell T, Richard H, Beauchamp G, *et al.* Joint inflammation increases glucosamine levels attained in synovial fluid following oral administration of glucosamine hydrochloride. Osteoarthritis Cartilage 2009;17:228-34.
58. Vangsness CT, Jr., Spiker W, Erickson J. A review of evidence-based medicine for glucosamine and chondroitin sulfate use in knee osteoarthritis. Arthroscopy 2009;25:86-94.
59. McAlindon T. Why are clinical trials of glucosamine no longer uniformly positive? Rheum Dis Clin North Am 2003;29:789-801.
60. Lems WF, Bijlsma JW. [Efficacy of dietary supplements in patients with osteoarthritis: the doubt persists] Effectiviteit van voedingssupplementen bij artrose: de twijfel blijft. Ned Tijdschr Geneeskd 2006;150:1105-7.

61. Bijlsma JW, Lafeber FP. Glucosamine sulfate in osteoarthritis: the jury is still out. Ann Intern Med 2008; 148:315-6.
62. Pelletier JP, Martel-Pelletier J. DMOAD developments: present and future. Bull NYU Hosp Jt Dis 2007; 65:242-8.
63. Qvist P, Bay-Jensen AC, Christiansen C, Dam EB, Pastoureau P, Karsdal MA. The disease modifying osteoarthritis drug (DMOAD): Is it in the horizon? Pharmacol Res 2008;58:1-7.

Chapter 8

Nederlandse samenvatting

Artrose, in de volksmond ook wel ‘kraakbeenslijtage’ genoemd, zal in een vergrijzende bevolking een wereldwijd probleem vormen. Zowel op sociaaleconomisch als op het persoonlijke vlak zal dit in de toekomst een groter aantal mensen raken. De term ‘kraakbeenslijtage’ is feitelijk onjuist. Dit impliceert namelijk dat het kraakbeen door het gebruik simpelweg zou slijten, terwijl artrose een veel complexer ziektebeeld is. Behalve het kraakbeen zijn bij artrose ook het onderliggende bot en het slijmvlies van het gewricht, het zogenaamde synovium, aangedaan. Het is een aandoening van alle componenten waaruit een gewricht is opgebouwd, want ook de structuren rondom het gewricht, zoals de banden en spieren, raken betrokken bij het ziekteproces van artrose.

De behandelingsmodaliteiten bij artrose bestaan tot nu toe uit het bestrijden van de symptomen door het gebruik van medicijnen of het volgen van oefenprogramma’s. Indien dit onvoldoende effect op de klachten heeft, is de laatste mogelijkheid om de pijn te verminderen en de beweeglijkheid te behouden het vervangen van een versleten gewricht door een kunstgewricht, een zogenaamde endoprothese. Middelen om het beloop van het ziekteproces van artrose te beïnvloeden zijn niet voorhanden. In het begin van de 21ste eeuw verschenen publicaties dat glucosamine (GlcN), een lichaamseigen stof die als voedingssupplement verkrijgbaar is, mogelijk wel positieve effecten zou hebben op het beloop van artrose bij patiënten.

Het doel van dit proefschrift is meer inzicht te verkrijgen in het werkingsmechanisme van GlcN bij artrose om daarmee een advies uit te kunnen brengen over het gebruik van dit voedingssupplement door patiënten die lijden aan artrose.

Hoofdstuk 1 is de algemene introductie. Hierin wordt beschreven dat een synoviaal gewricht opgebouwd is uit twee scharnierende botten die aan de uiteinden bedekt zijn met gewrichtskraakbeen. Dit geheel wordt omgeven door een gewrichtskapsel, waarvan de binnenbekleding het synovium genoemd wordt (gewrichtslijmvlies). Artrose is een ziekte van het gehele gewricht, waarbij dus alle bovengenoemde onderdelen in meer of mindere mate zijn aangedaan. De huidige behandelingsmogelijkheden van artrose bestaan grofweg uit conservatieve (niet-operatieve) en operatieve opties. Al deze behandelingen bestrijden de symptomen van artrose, maar geen van hen geneest de artrose zelf. Daarom duurt de zoektocht naar een ziektemodificerend middel nog altijd voort. Van GlcN wordt beweerd dat dit ziektemodificerende eigenschappen bezit. GlcN is een voedingssupplement dat van nature in ons lichaam voorkomt en gevormd wordt uit glucose. Na fosforylering en conversie naar fructose-6-fosfaat, wordt glucosamine-6-fosfaat gevormd door middel van een amidotransferase reactie met glutamine als amino donor. Sommige onderzoekers beweren dat GlcN effectief is, omdat het direct als bouwstof voor de extracellulaire matrix (ECM) van kraakbeen gebruikt kan worden. In de literatuur zijn voor deze theorie zowel bewijzen voor als tegen terug te vinden. Andere onderzoekers beweren dat GlcN interfereert met de enzymatische ECM afbraak en op die manier zijn effect sorteert. Tot slot wordt het doel en de opbouw van het proefschrift weergegeven.

In **hoofdstuk 2** wordt beschreven wat de effecten in kweek zijn van glucosamine-sulfaat (GlcN-S) en glucosamine-hydrochloride (GlcN-HCl) op kraakbeen afkomstig van patiënten die een totale knieprothese kregen wegens artrose. In deze studie werd de expressie van genen betrokken bij anabole en catabole processen in het kraakbeen bestudeerd. Tevens werd het kweekmedium onderzocht om de invloed van GlcN op de totale matrix metalloproteïnase (MMP) activiteit te bepalen. Toevoeging van 5 mM GlcN leidde tot een significant lagere expressie ten opzichte van de controle conditie van aggrecan en collageen type 2, genen die coderen voor twee belangrijke componenten van de ECM. Verder zorgde GlcN voor een significant lagere expressie van aggrecanase-1, ook wel bekend als A Desintegrin And a Metalloproteinase with TromboSpondin motifs 4 (ADAMTS4) en MMP3, die een rol spelen bij de afbraak van de ECM. Toevoeging van 5 mM GlcN-S leidde ook nog tot een significante verlaging van de expressie van ADAMTS5 en Tissue Inhibitor of MMP 3 (TIMP3) ten opzichte van de controle conditie. Analyse van het kweekmedium toonde dat, in vergelijking met de controle conditie, de totale MMP activiteit significant was afgenomen na toevoeging van 5 mM GlcN-HCl. Op basis van de bevindingen in dit weefselkweekmodel met kraakbeen afkomstig van mensen met artrose werd geconcludeerd dat GlcN in staat lijkt om de enzymatische afbraak van de ECM af te remmen. Gezien de verlaging van de expressie van genen verantwoordelijk voor ECM opbouw lijkt het echter niet waarschijnlijk dat GlcN voor herstel van beschadigd kraakbeen kan zorgen. De mogelijke kraakbeenbeschermende eigenschappen van GlcN *in vivo* zijn dus eerder gebaseerd op het remmen van verdere enzymatische ECM afbraak dan op het herstel van reeds aangedaan kraakbeen.

Om het werkingsmechanisme van GlcN verder te ontrafelen is in **hoofdstuk 3** gebruik gemaakt van een kweekmodel met kraakbeencellen van kalveren die gezaaid werden in een driedimensionale matrix. Hiervoor werd gebruik gemaakt van alginaat waarvan bollen gevormd werden. Deze bollen met kraakbeencellen werden vervolgens in kweek gebracht, waarna er op verschillende tijdstippen GlcN-S, GlcN-HCl of N-acetyl-glucosamine (GlcN-Ac) aan werd toegevoegd. In deze experimenten werd bestudeerd wat de invloed is van de metabole toestand van een kraakbeencel op de effecten van het toevoegen van GlcN. Allereerst werd onderzocht wat het effect van GlcN is op kraakbeencellen, die in een situatie verkeren van balans tussen ECM aanmaak en afbraak. Dit werd bereikt door de alginaatbollen eerst drie weken voor te kweken en daarna gedurende twee weken de GlcN derivaten toe te voegen. Hierbij had alleen GlcN-Ac een klein positief effect op de totale hoeveelheid glycosaminoglycanen (GAG), hetgeen een maat is voor de opgebouwde ECM. GlcN-S en GlcN-HCl hadden in deze situatie geen effect. Een hoofdzakelijk anabole situatie, dus waar de ECM nog opgebouwd moest worden, werd nagebootst door reeds na drie dagen primaire voorkweek te starten met toevoegen van de experimentele condities gedurende twee weken. De resultaten van deze experimenten lieten zien dat GlcN-S en GlcN-HCl toevoeging er voor zorgt dat de hoeveelheid GAG significant kleiner wordt ten opzichte van de controle conditie. GlcN-Ac had in deze situatie geen effect. Om een

katabole situatie te creëren werd gebruik gemaakt van een model waarbij interleukine-1β werd toegevoegd. Het katabole effect van interleukine-1β (verdere vermindering van de hoeveelheid GAG) werd voorkomen door een voorbehandeling van de kraakbeencel-alginaatbollen met GlcN-HCl. Om de relatie tussen glucose en GlcN concentratie te onderzoeken werd ook nog een experiment uitgevoerd met oplopende glucose concentraties bij gelijkblijvende GlcN concentraties. De verminderde GAG hoeveelheid ten gevolge van GlcN toevoeging in de anabole situatie werd teniet gedaan door hogere glucose concentraties. Op basis van deze experimenten werd geconcludeerd dat GlcN-S en GlcN-HCl, maar niet GlcN-Ac, zowel anabole als katabole processen in kraakbeen kunnen remmen. Dit zou mogelijk gebaseerd kunnen zijn op een interactie met het glucosemetabolisme, omdat de door GlcN veroorzaakte effecten afnamen bij een toegenomen glucose concentratie. De mogelijke effecten van GlcN op kraakbeen zijn dus waarschijnlijk meer gebaseerd op bescherming tegen afbraak van de ECM dan op de productie van nieuwe ECM.

Aangezien kraakbeen geen zenuwuiteinden bevat kunnen de effecten van GlcN, die in de literatuur beschreven zijn op pijn, niet verklaard worden op basis van effecten op kraakbeen. Eerder is al wel beschreven dat injecties met hyaluronzuur in het kniegewricht leidde tot pijnvermindering bij patiënten met artrose. Voor de vorming van hyaluronzuur is GlcN nodig als één van de bestanddelen van deze stof. Hyaluronzuur wordt in een gewricht gevormd door het synovium. In **hoofdstuk 4** wordt daarom met een synoviumweefselkweekmodel onderzocht of toevoeging van GlcN leidt tot een verhoging van de hyaluronzuur concentratie. Het synoviumweefsel werden geoogst tijdens totale knieprothese operaties wegens artrose. Vervolgens werden deze stukjes weefsel in kweek gebracht en werd GlcN-HCl of GlcN-Ac toegevoegd. In het kweekmedium werd de totale hoeveelheid hyaluronzuur gemeten. In het synoviumweefsel werd de genexpressie van de enzymen verantwoordelijk voor de vorming van hyaluronzuur, hyaluronzuursynthase 1, 2 en 3, bepaald. Toevoeging van 0.5 of 5 mM GlcN-HCl liet een significante toename in hoeveelheid hyaluronzuur zien (2-4 maal) in vergelijking met de controle conditie. GlcN-Ac had hierop geen effect. Ook glucose had een effect op de hyaluronzuurproductie echter alleen bij hoge concentratie. Er werd geen effect gevonden op de genexpressie van hyaluronzuursynthase na de toevoeging van GlcN.

De data van deze studie suggereren dat het pijnverminderende effect toegeschreven aan GlcN verklaard zou kunnen worden door een toegenomen hoeveelheid hyaluronzuur.

In **hoofdstuk 5** worden de resultaten beschreven van een subgroep analyse van een cohort van 222 patiënten met heupartrose, uit de huisartspraktijk, die voldeden aan de criteria van het American College of Rheumatology. Deze patiënten gebruikten gedurende een periode van twee jaar eenmaal daags oraal 1500 mg GlcN-S of placebo. De exacte opzet van deze gerandomiseerde, placebo gecontroleerde studie is terug te vinden in de **appendix** van dit proefschrift. De vooraf gedefinieerde subgroepen waren gebaseerd op type artrose (gelokaliseerd alleen in

de heup of gegeneraliseerd, dus in meerdere gewrichten) en op radiologische ernst (Kellgren and Lawrence 1 of ≥ 2). Tevens werd additionele exploratieve subgroep analyse verricht naar groepen op basis van baseline gewrichtspleet en aanwezigheid van knie artrose op baseline, pijnniveau en gebruik van pijnmedicatie. Na twee jaar werd in de vooraf gedefinieerde subgroepen geen verschil gevonden tussen GlcN-S en placebo voor de primaire uitkomstmaten pijn, functie (gemeten met de Western Ontario and McMaster Universities osteoarthritis index) en gewrichtspleetversmalling. Ook in de additionele exploratieve subgroep analyse werd geen verschil gevonden tussen GlcN-S en placebo. De conclusie was derhalve dat GlcN-S niet significant beter was dan placebo in het verminderen van de symptomen en progressie van heupartrose in deze subgroepen. Dit was conform de bevindingen bij de analyse van het totale cohort. Glucosamine lijkt dus op heupartrose geen effect te hebben.

In hoofdstuk 2, 3 en 4 werd onderzoek gedaan met behulp van kweekmodellen naar het werkingsmechanisme van GlcN bij artrose. Hoofdstuk 5 beschreef de effecten van oraal GlcN gebruik gedurende twee jaar op patiënten. Hierbij werd met name gekeken naar de effecten op de symptomen en de tekenen van progressie van de artrose. In een poging om deze twee onderzoeksmethoden te combineren, werd er in **hoofdstuk 6** onderzocht of het mogelijk is om effecten op genexpressie van kraakbeen en bot na een periode van preoperatief GlcN versus placebo gebruik te meten in patiënten met heup artrose. Net als in hoofdstuk 2 werd gekeken naar genen betrokken bij anabole en katabole processen. Het effect van preoperatief GlcN-S gebruik op genexpressie van kraakbeen en bot werd onderzocht op materiaal afkomstig van patiënten die tijdens de studie beschreven in hoofdstuk 5, een totale heupprothese kregen. In deze exploratieve pilot studie werd er een indicatie gevonden dat er voor subchondraal bot een verminderde expressie van MMP13 bestaat bij patiënten die GlcN-S gebruikten ten opzichte van de patiënten die placebo gebruikt hadden. Er was geen indicatie dat er een effect was op de genexpressie van kraakbeen voor GlcN-S ten opzichte van placebo. In de tweede exploratieve studie beschreven in hoofdstuk 6, gebruikten patiënten die op de opnamelijst stonden voor een totale heupprothese in het Erasmus MC Rotterdam, eenmaal daags 1500 mg GlcN-HCl of placebo gedurende 8-10 weken preoperatief. In deze exploratieve pilot studie werd peroperatief de genexpressie van het kraakbeen bepaald. Hierbij werd een indicatie gevonden dat de genexpressie van aggrecan peroperatief significant lager was bij de patiënten die GlcN-HCl gebruikt hadden ten opzichte van de placebogroep. Tevens werd in deze GlcN-HCl studie het nog resterende kraakbeen van de heupkoppen gebruikt voor kweekexperimenten, waarin toevoeging van GlcN-HCl vergeleken werd met een controle conditie. In deze kweekexperimenten waren er indicaties dat collageen type 2 en aggrecan significant lager tot expressie kwamen bij de 0.5 mM GlcN-HCl conditie in vergelijking met de bijpassende controle conditie. Bij nadere analyse lijkt dit effect met name duidelijk naar voren te komen bij de kraakbeensamples afkomstig van de patiënten die preoperatief GlcN-HCl gebruikt hadden. Gezien het feit dat dit een exploratieve pilot studie betreft, werden de

peroperatieve genexpressie data van de patiënten die placebo gebruikt hadden, in zowel de GlcN-S als de GlcN-HCl studie, tevens gebruikt om groepsgroottes te berekenen voor toekomstige vervolgonderzoeken. Geconcludeerd werd dat het mogelijk is om effecten op genexpressie in kraakbeen en bot na een periode van preoperatief GlcN gebruik te meten. Tevens lijkt het dat GlcN-HCl *in vivo* het kraakbeen mogelijk beïnvloedt, waardoor het kraakbeen in kweekexperimenten gevoeliger is voor de toevoeging van GlcN-HCl. Dit laatste is iets waar, bij de planning van toekomstige experimenten met humaan kraakbeen, wellicht rekening mee moet worden gehouden. De gevonden trend van een lagere genexpressie van aggrecan en collageen type 2 voor GlcN-HCl in vergelijking met placebo, zal in een toekomstige vervolgstudie gerepliceerd moeten worden. De noodzakelijke groepsgrootte voor een dergelijke studie bedraagt 13 to 17 patiënten per groep.

Hoofdstuk 7 is de samenvatting en algemene discussie, waarin de bevindingen van de voorgaande hoofdstukken opgesomd en in relatie met elkaar en de literatuur gebracht worden. Naast het bespreken van de belangrijkste beperkingen van de onderzoeken in dit proefschrift, worden er aanbevelingen gedaan waar, bij het doen van toekomstig onderzoek naar ziekte modificerende middelen bij artrose, rekening mee gehouden zou moeten worden. Deze aanbevelingen zijn:

1) Bij kweekexperimenten dienen de effecten van de te onderzoeken substantie bepaald te worden op alle onderdelen van een synoviaal gewricht, te weten bot, kraakbeen en synovium
2) Bij het opzetten van een kweekexperiment moet de concentratie van de te onderzoeken substantie zo dicht mogelijk bij die van de synoviaal vloeistof liggen, gegeven dat deze data voor handen zijn.
3) Bij experimenten waarbij gebruik gemaakt wordt van chondrocyten in een driedimensionale omgeving kan de toestand van de ECM de uitkomst beïnvloeden. Daarom moet vastgesteld worden of de chondrocyten zich in een anabole omgeving of in een omgeving met balans tussen ECM aanmaak en afbraak bevinden.
4) Als een artrose model gebruikt wordt, om zodoende een katabole omgeving te creëren, lijkt het gebruik van humaan artrose materiaal (kraakbeen, synovium) het meest voor de hand liggend om de *in vivo* situatie zo dicht mogelijk te benaderen. Wij hebben laten zien dat het mogelijk is om humaan weefsel te gebruiken om de effecten van ziektemodificerende artrose middelen te onderzoeken.
5) Bij de selectie van patiënten van wie het materiaal gebruikt gaat worden in dergelijke experimenten, zou er rekening gehouden moeten worden met het eerdere gebruik van middelen die zonder recept verkrijgbaar zijn en mogelijk ziektemodificerende effecten op artrose hebben. Het materiaal van deze patiënten zou wellicht uitgesloten moeten worden van het gebruik in kweekexperimenten.

6) Om het conflict dat ontstaat bij het vertalen van de kweekmodel situatie naar de klinische effectiviteit te omzeilen, kunnen de effecten van ziektemodificerende artrose middelen ook onderzocht worden in patiënten. Maar, bij het ontwerp van dergelijke studie is het van belang geïnformeerd te zijn over het gebruik van gelijksoortige, zonder recept verkrijgbare ziektemodificerende artrose middelen en het zou verstandig kunnen zijn deze patiënten bij een dergelijke trial te excluderen.
7) Bij het doen van onderzoek naar de effecten van ziektemodificerende artrose middelen op patiënten, is één methode om zoveel mogelijk materiaal voor analyse te verkrijgen, het gebruik maken van restmateriaal verkregen bij gewrichtsvervangende operaties. Om dit voor elkaar te krijgen moeten de patiënten de ziektemodificerende artrose middelen gebruiken binnen een placebo gecontroleerde trial. Om de vergelijkbaarheid van de resultaten te vergroten, is het van belang dat de duur van het gebruik van het actieve middel, dan wel de placebo, zo gestandaardiseerd mogelijk is.
8) Genexpressie kan als uitkomstparameter zeer waardevol zijn, maar aanvullende gegevens over de uiteindelijke verandering van de producten waarvoor deze genen coderen, zouden ook gegeven moeten worden.

Appendices

Dankwoord

Curriculum Vitae

PhD Portfolio

List of Publications

The effect of glucosamine sulphate on osteoarthritis: design of a long-term randomised clinical trial

DANKWOORD

Hier is hij dan: mijn proefschrift! Alhoewel, 'mijn' proefschrift……
Zonder de bijdrage, hulp en ondersteuning van velen, op welke manier dan ook, was ik niet tot dit resultaat gekomen. Vandaar dat ik een aantal mensen hiervoor wil bedanken. Mocht ik daarbij onverhoopt iemand vergeten: ook jij hartelijk bedankt.

Prof.dr. J.A.N. Verhaar, promotor, beste professor, bijna 10 jaar geleden was u secretaris van de examencommissie tijdens mijn artsexamen, nu mijn promotor en tussendoor ook nog mijn opleider. Bij mijn aanstelling destijds op het lab was één van de eerste regels die u mij vertelde dat er op wetenschappelijk vlak op basis van gelijkwaardigheid met elkaar gecommuniceerd diende te worden, iets wat ik altijd erg gewaardeerd heb. Manuscript na idee na vraag, hoe belangrijk of onbeduidend ook, altijd kwam er een gefundeerde, kritische reactie en meestal binnen 24 uur. Dank dat u mij de mogelijkheid en ondersteuning heeft geboden om dit promotieonderzoek te laten resulteren in het huidige proefschrift en dat ik mijn opleiding tot orthopaedisch chirurg in de Rotterdamse ROGO mag volgen.

Prof.dr.ir. H. Weinans, promotor, beste Harrie, jouw wetenschappelijk enthousiasme, over welk onderzoek dan ook, lijkt onuitputtelijk en dat bewonder ik. Elk manuscript, ongeacht de hoeveelste versie, werd telkens met een frisse invalshoek van commentaar voorzien. In onze discussies gingen de sprongen van wit naar zwart naar grijs soms zo snel dat ik wel eens moeite had om je bij te houden, maar uiteindelijk rolde er wel altijd iets uit dat beter was dan voorheen. Dat mijn proefschrift geworden is zoals het nu is, heb ik mede aan jou te danken.

Dr. G.J.V.M. van Osch, co-promotor, beste Gerjo, zonder jou was dit proefschrift er misschien wel helemaal niet geweest; of zeker niet in de huidige vorm. Jij hebt mij met onvermoeibare inzet van het begin af aan bij mijn promotieonderzoek met raad en daad bijgestaan. Op tijden als de voortgang wat leek te stagneren, wist jij de boel weer vlot te trekken. Jouw manier om elke keer weer op een andere wijze naar een dataset te kunnen kijken en er een boodschap uit te halen is een kunst op zich. Ik had me werkelijk geen betere begeleider kunnen wensen.

De laboratoriumstudies had ik nooit kunnen doen zonder de ondersteuning van diverse mensen op het orthopaedielab. Pieter, bedankt voor je hulp bij de uitvoer en analyses van de experimenten in het begin van mijn onderzoek. Rodi, behalve dat het leuk was om jou te begeleiden bij je afstudeeronderzoek, is het succesvolle verloop van de IL-1β experimenten mede aan jou te danken. Joy, Carola en Predrag, materiaal oogsten is één, RNA opwerken is weer wat anders. Heel erg bedankt hiervoor. In het bijzonder wil ik Wendy enorm bedanken voor de assistentie, zelfstandige uitvoering en analyse van alle experimenten die ik ook maar bedacht, zeker die van na mijn vertrek uit het lab.

Holger Jahr, jouw moleculair biologische kennis is van grote waarde geweest voor dit proefschrift. Dank voor de wijze waarop je de vaak moeilijke materie helder wist uit te leggen en voor de bijdragen die je aan mijn artikelen hebt geleverd. Ook het vertalen van voor mij onbegrijpelijke woorden op één of ander Duits BMW forum kwam zo nu en dan goed van pas.

Ook de overige collega's van het lab, Jarno, Justus, Erik, Luuk, Marieke, Jaqueline, Erwin, Judd, Nicole, Esther, Yvonne B-J, Yvonne S, Ruud, Sander en Irene, die ervoor gezorgd hebben dat ik in die ruim twee jaar een toptijd heb gehad, wil ik hiervoor hartelijk bedanken.

Mijn paranimfen, beste Patrick, al vanaf het eerste jaar geneeskunde zijn we samen opgetrokken. De eerste serieuze werkzaamheden op het onderzoeksvlak heb ik samen met jou verricht tijdens ons gezamenlijke afstudeeronderzoek op de afdeling anatomie. Fijn dat je je drukke praktijk wil verlaten om mij bij de promotie bij te staan.
Beste Jarno, als je twee jaar lang op het lab tegenover elkaar moet zitten, is het maar goed dat je een beetje met elkaar overweg kan. Gelukkig bleken we genoeg gezamenlijke interesses te hebben, om niet altijd over het werk te hoeven praten. Bedankt dat je me tijdens mijn promotie als paranimf en één maand 'oudere' assistent bij wil staan.

De collega's van de afdeling huisartsgeneeskunde wil ik hartelijk bedanken voor de plezierige en vruchtbare samenwerking. Sita, dank voor je kritische beoordelingen van en bijdragen aan mijn manuscripten. Max, inmiddels collega bij de Orthopaedie, bedankt voor de Kellgren en Lawrence ondersteuning. Beste Rianne, 'binnenkort' is dan toch nog bijna een jaar geworden. Bedankt dat ik actief mocht meedenken en bijdragen aan de GOAL trial. Zonder jou geen hoofdstuk 5.

Alle secretaresses, polikliniekmedewerksters, verpleegkundigen van de verpleegafdeling, de OK en de polikliniek van de Orthopaedie van het Erasmus MC, bedankt voor jullie ondersteuning en betrokkenheid bij de glucosamine-hydrochloride patiëntenstudie. Marein, bedankt voor het werk dat je hebt verricht om het onderzoeksprotocol door de METC te loodsen. Simone, jij speciaal bedankt voor alle dingen die je in het laatste traject van mijn promotie hebt geregeld.

Jeroen de Groot en Yvonne, bedankt voor jullie hulp bij de MMP assays. Carl Verkoelen, ondanks dat je er niet meer bent, dank voor het enthousiaste en kritische meedenken bij de hyaluronzuur analyses destijds. Charlie Laffeber, ook jij bedankt voor je ondersteuning bij de hyaluronzuur assays.

Zonder patiënten geen onderzoek. Dank aan al die patiënten die deelgenomen hebben aan de klinische studies in dit proefschrift. Tevens gaat mijn dank uit naar de orthopaedisch chirurgen in de regio Rotterdam, die zo bereid en gastvrij waren ons te ontvangen op hun operatiekamers om mee te werken aan het afstaan van de heupkoppen van patiënten uit de

glucosamine-sulfaat studie. Het aantal geoogste heupkoppen was nooit zo hoog geweest, als de collega's van het lab het ophalen niet van mij hadden overgenomen toen ik met mijn vooropleiding begon. Allemaal dank hiervoor.

De stafleden van de Orthopaedie van het Erasmus MC Rotterdam, Ad Diepstraten, Frans van Biezen, Rien Heijboer, Luuk de Klerk, Gert Bessems, Duncan Meuffels, Koen Bos, de orthopaeden van het St. Elisabeth ziekenhuis te Tilburg, dokter Bonnet, Jan de Waal Malefijt, Jacob Caron, Carel Diekerhof, Taco Gosens, Igor van den Brand, evenals Dr. L.P.S. Stassen en zijn collega's, verantwoordelijk voor mijn chirurgische vooropleiding in het Reinier de Graaf gasthuis te Delft, en al mijn collega-assistenten in de loop van al die jaren, wil ik bedanken voor de prima opleidingstijd en de ruimte die ik daarnaast gekregen heb om dit promotieonderzoek te kunnen voltooien. De interesse die jullie toonden, heb ik altijd erg op prijs gesteld.

Ron te Slaa, Hennie Verburg, Rolf Bloem, Ruud Deijkers en Dieu Donné Niesten, de orthopaeden van het Reinier de Graaf gasthuis te Delft, die mij destijds als groentje de kans geboden hebben om de eerste schreden in de orthopaedie te zetten, wil ik hiervoor hartelijk bedanken. Zonder jullie was dit vervolgtraject er wellicht niet geweest. Ron, jammer dat je dit niet meer mee mag maken.

Heren van de 'Oostenrijkse wijnclub', helaas heb ik het de laatste tijd wat af moeten laten weten vanwege de afronding van mijn boekje, maar de (half)jaarlijkse, voor sommigen onbegrijpelijke ("Ga je skiën dan?") tripjes naar de Steiermark waren altijd een welkome, onbezorgde mogelijkheid om de opleidings- en promotieperikelen te vergeten en even bij te tanken.

Mijn broertje en schoonzus, Martijn en Hadewig en mijn schoonfamilie, Karel en Henny, Loes en Pièrre met Max, bedankt voor jullie belangstelling en morele ondersteuning op de momenten dat het niet altijd even makkelijk ging. Gelukkig is het proefschrift af, zodat ik weer wat meer tijd heb voor jullie en de nieuwe aanwinst(en).

Pa en ma, na een onbezorgde jeugd en een dito studententijd hebben jullie me altijd op alle mogelijke manieren ondersteund om te kunnen doen en laten wat ik wilde. De manier waarop jullie ons hebben gestimuleerd voor het beste te gaan, heeft er voor gezorgd dat ik zover ben gekomen.

Tot slot is er nog een persoon zonder wie ik dit nooit had kunnen volbrengen. Lieve Dorien, het zijn soms heftige tijden geweest, maar ondanks dat je het niet altijd even gemakkelijk had, heb je me nooit een strobreed in de weg gelegd en heb je me altijd gesteund om te doen wat ik wilde bereiken. Hopelijk komt met het voltooien van mijn proefschrift de bureaustoel - bank ratio weer eens onder de één uit. Ik hou van je.

CURRICULUM VITAE

Elian Uitterlinden werd geboren op 22 september 1975 te Dordrecht. Na het behalen van het VWO diploma in 1993 aan het Titus Brandsma college in zijn geboorteplaats ging hij in hetzelfde jaar, wegens uitloting voor de studie geneeskunde, econometrie studeren aan de Erasmus Universiteit te Rotterdam. Eén jaar later werd hij wel ingeloot en zo kon in 1994, eveneens in Rotterdam, de studie geneeskunde aangevangen worden. In 1998 werd het doctoraalexamen en vervolgens in 2000 het artsexamen behaald. Van oktober 2000 tot oktober 2002 is hij werkzaam geweest als AGNIO Chirurgie / Orthopaedie en later als AGNIO Orthopaedie en Traumatologie in het Reinier de Graaf gasthuis te Delft. Hierna begon hij als arts-onderzoeker op het Orthopaedic Research Laboratory van de Erasmus Universiteit te Rotterdam met zijn promotieonderzoek onder leiding van prof.dr. J.A.N. Verhaar, prof.dr.ir. H. Weinans en dr. G.J.V.M. van Osch. In februari 2005 begon hij met de chirurgische vooropleiding in het Reinier de Graaf gasthuis te Delft (Opleider: dr. L.P.S. Stassen). In februari 2007 werd de opleiding tot orthopaedisch chirurg door hem aangevangen in het Erasmus MC te Rotterdam (Opleider: prof.dr. J.A.N. Verhaar). Na zijn perifere stage orthopaedie van juli 2008 tot juli 2009 doorgebracht te hebben in het St. Elisabeth ziekenhuis te Tilburg (Opleider: dr. J. de Waal Malefijt), zal hij de verdere specialisatie tot orthopaedisch chirurg afronden in het Erasmus MC te Rotterdam.

PHD PORTFOLIO

Courses

Cursus "schrijven in Engels" (NTVG) 2002

Cursus Good Clinical Practice (ICH, training en advies) 2003

Conferences and presentations

Podium presentations

Altered gene-expression in osteoarthritic subchondral bone. Dutch Society for Matrix Biology (NVMB), Lunteren, The Netherlands 2003

Altered gene expression in osteoarthritic subchondral bone. Dutch Society for Calcium and Bone Metabolism (NVCB), Papendal, The Netherlands 2003

Effect of glucosamine derivatives on cultured chondrocytes; proposal for a mechanism of action. Dutch Society for Matrix Biology (NVMB), Lunteren, The Netherlands 2004
Awarded Pauline van Wachem best podium presentation award

Glucosamine en Artrose. 'Beweging in onderzoek'; patiëntencongres Reumafonds, Rotterdam, The Netherlands 2004
Invited lecture

Effect of glucosamine derivatives on chondrocytes cultured in alginate; proposal for a mechanism of action. 5th World Congress of the International Cartilage Repair Society (ICRS), Gent, Belgium 2004

Glucosamine remt de afbraak en aanmaak van extracellulaire matrix in kraakbeen explants van artrose patiënten. Annual meeting Dutch Orthopaedic Society (NOV), Veldhoven, The Netherlands 2005

Glucosamine in OsteoArthritis: Long-term effectiveness. Annual meeting Dutch Orthopaedic Society (NOV), Rotterdam, The Netherlands 2007

Effectiveness of glucosamine sulphate in hip osteoarthritis: A long-term double-blind randomised clinical trial. Annual Congress European League Against Rheumatism (EULAR), Barcelona, Spain 2007

Glucosamine: de klinische stand van zaken. Belgian Hip Society, Leuven, Belgium 2008

Glucosamine Onderzoek bij Artrose: Lange termijn effectiviteit. Dutch College of General Practitioners (NHG), Rotterdam, The Netherlands 2008

Poster presentations

Lange termijn effectiviteit van glucosaminesulfaat bij heupartrose: design van een RCT. Dutch College of General Practitioners (NHG), Rotterdam, The Netherlands 2005

Effects of glucosamine on chondrocytes in anabolic and catabolic conditions; indications for interference with chondrocyte energy management. 52nd Annual meeting Orthopaedic Research Society (ORS), Chicago, USA 2006

Glucosamine inhibits extracellular matrix degradation and synthesis in human osteoarthritic cartilage explants. 52nd Annual meeting Orthopaedic Research Society (ORS), Chicago, USA 2006

Glucosamine reduces catabolic and anabolic processes in chondrocytes; Indications for interference with intracellular energy management. 11th World Congress on Osteoarthritis (OARSI), Prague, Czech Republic 2006

Glucosamine sulphate in hip OA, the GOAL study: subgroup analyses. 12th World Congress on Osteoarthritis (OARSI), Fort Lauderdale, USA 2007

Teaching activities

Author self-study assignment "Voedingssupplementen en Artrose" 4th year medical students 2004

Lectures and practicals "Integratie Vaardigheids Onderwijs (IVO)" 4th year medical students 2004 2007 2009

Supervising Master's thesis "Effect van glucosamine in een celkweek model voor artrose" R.M. Horstman, 4th year medical student, 21 weeks 2004

LIST OF PUBLICATIONS

1) Rozendaal RM, **Uitterlinden EJ**, van Osch GJ, Garling EH, Willemsen SP, Ginai AZ, Verhaar JA, Weinans H, Koes BW, Bierma-Zeinstra SM. Effect of glucosamine sulphate on joint space narrowing, pain and function in patients with hip osteoarthritis; subgroup analyses of a randomized controlled trial. Osteoarthritis Cartilage 2009 Apr;17(4):427-32

2) Rozendaal RM, Koes BW, van Osch GJ, **Uitterlinden EJ**, Garling EH, Willemsen SP, Ginai AZ., Verhaar JA, Weinans H, Bierma-Zeinstra SM. The effect of glucosamine sulfate on the progress of hip arthritis. Huisarts Wet. 2008; 51(11):535-41

3) **Uitterlinden EJ**, Koevoet JL, Verkoelen CF, Bierma-Zeinstra SM, Jahr H, Weinans H, Verhaar JA, van Osch GJ. Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants. BMC Musculoskelet Disord 2008 Sep 11; 9:120

4) Rozendaal RM, Koes BW, van Osch GJ, **Uitterlinden EJ**, Garling EH, Willemsen SP, Ginai AZ, Verhaar JA, Weinans H, Bierma-Zeinstra SM. Effect of glucosamine sulfate on hip osteoarthritis: a randomized trial. Ann Intern Med 2008 Feb 19; 148(4):268-77

5) Rozendaal RM, Koes BW, van Osch GJ, **Uitterlinden EJ**, Garling EH, Ginai AZ, Verhaar JA, Weinans H, Bierma-Zeinstra SM. Effectiveness of glucosamine sulphate in hip osteoarthritis: A long-term double-blind randomised clinical trial [ISRCTN54513166]. Ann Rheum Dis 2007;66(Suppl II):59

6) **Uitterlinden EJ**, Jahr H, Koevoet JL, Bierma-Zeinstra SM, Verhaar JA, Weinans H, van Osch GJ. Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate. Osteoarthritis Cartilage 2007 Nov; 15(11):1267-74

7) **Uitterlinden EJ**, Jahr H, Koevoet JL, Jenniskens YM, Bierma-Zeinstra SM, Degroot J, Verhaar JA, Weinans H, van Osch GJ. Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. Osteoarthritis Cartilage 2006 Mar; 14(3):250-7
Awarded Top 10 Cited Paper 2006-2008 Osteoarthritis and Cartilage

8) van Cingel RE, Kleinrensink GJ, **Uitterlinden EJ**, Rooijens PP, Mulder PG, Aufdemkampe G, Stoeckart R. Repeated ankle sprains and delayed neuromuscular response: acceleration time parameters. J Orthop Sports Phys Ther 2006 Feb; 36(2):72-9

9) Rozendaal RM, Koes BW, Weinans H, **Uitterlinden EJ**, van Osch GJ, Ginai AZ, Verhaar JA, Zeinstra SM. The effect of glucosamine sulphate on osteoarthritis: design of a long-term randomised clinical trial [ISRCTN54513166]. BMC Musculoskelet Disord 2005 Apr 26; 6:20

10) van Cingel RE, Kleinrensink GJ, Rooijens PP, **Uitterlinden EJ**, Aufdemkampe G, Stoeckart R. Learning effect in isokinetic testing of ankle invertors and evertors. Isokinetics and Exercise Science 2001; 9(4):171-7

Appendix

The effect of glucosamine sulphate on osteoarthritis: design of a long-term randomised clinical trial

R.M. Rozendaal, B.W. Koes, H. Weinans, E.J. Uitterlinden, G.J.V.M. van Osch,
A.Z. Ginai, J.A.N. Verhaar, S.M.A. Bierma-Zeinstra

BMC Musculoskelet Disord 2005 Apr 26; 6:20

ABSTRACT

Background

Pharmacological treatment for osteoarthritis (OA) can be divided into two groups: symptom-modifying drugs and disease-modifying drugs. Symptom-modifying drugs are currently the prescription of choice for patients with OA, as disease-modifying drugs are not yet available in usual care. However, there has recently been a lot of debate about glucosamine sulphate (GlcN-S), a biological agent that is thought to have both symptom-modifying and disease-modifying properties. This assumption has yet to be proved.

The objective of this article is to present the design of a blind randomised clinical trial that examines the long-term symptom-modifying and disease-modifying effectiveness of GlcN-S in patients with hip OA. This trial is ongoing and will finish in March 2006.

Methods/Design

Patients with hip OA meeting the ACR-criteria are randomly allocated to either 1500 mg of oral GlcN-S or placebo for the duration of two years. The primary outcome measures, which are joint space narrowing (JSN), and change in the pain and function score of the Western Ontario McMaster Universities Osteoarthritis index (WOMAC), are determined at baseline and after two years of follow-up during the final assessment. Intermediate measures at three-month intervals throughout the trial are used to study secondary outcome measures. Secondary outcome measures are changes in WOMAC stiffness score, quality of life, medical consumption, side effects and differences in biomarker CTX-II.

INTRODUCTION

Pharmacological treatment for osteoarthritis (OA) can be divided into two groups: symptom-modifying drugs and disease-modifying drugs [1]. Symptom-modifying drugs are at present the prescription of choice for patients with OA. Drugs in this group are: simple analgesics (such as acetaminophen) and non-steroidal anti-inflammatory drugs (NSAIDs). Both acetaminophen and NSAIDs are effective in relieving symptoms of OA. In more severe stages of the disease NSAIDs are more effective, however, they are also the cause of serious side effects [2].

Disease-modifying drugs (i.e., drugs which alter disease progression) are not yet available in usual care. Although there has recently been a lot of debate about some biological agents that are thought to have both symptom-modifying and disease-modifying properties, results from previous trials have not been convincing. Of these biological agents, glucosamine sulphate seems to be the most promising.

Glucosamine sulphate (GlcN-S) has been shown to be an effective symptom-modifying agent, with effect sizes ranging from moderate to high [3,4]. In four trials that compared GlcN-S and NSAIDs, GlcN-S was found to be as effective as, or slightly more effective than NSAIDs [4]. Together with the fact that no serious adverse events have been reported concerning GlcN-S [3,4] this implies that GlcN-S may be a good alternative to NSAIDs. However, due to publication bias and due to quality issues in the trials studying GlcN-S, it may well be that reported effect sizes are exaggerated. A more recent trial studying the effect of GlcN-S did not find a difference between GlcN-S and placebo [5]. Also, several other uncertainties exist concerning the symptom-modifying properties of GlcN-S. For example, most trials were only of short-term duration (e.g., mean 6.25 weeks [4]) and it is therefore not possible to draw conclusions about the long-term efficacy. Another problem is that the mechanism behind the improvement of symptoms due to GlcN-S is not known. If GlcN-S directly influences the remaining cartilage, it would seem plausible that the symptomatic effect is greater in people with mild to moderate OA than in people with more severe OA, because there is more cartilage remaining in the first group. However, this possible difference in effect between different stages of OA has not been tested yet in a randomised clinical trial (RCT). These uncertainties make further study into the magnitude of long-term symptom-modifying effects in different stages of the disease justified.

Concerning disease-modifying effects, two recent long-term (three years) trials in patients with knee OA did report some evidence that GlcN-S affected the progression of OA [6,7]. Progressive joint space narrowing in the narrowest medial compartment of the tibiofemoral joint was used to define progression of knee OA (as recommended by a task force of the OA research society [8]). Whereas joint space narrowing (JSN), had significantly progressed in the placebo groups, it had not in the groups that were taking GlcN-S. This implies that daily intake of GlcN-S acted against progression of OA. However, these results are controversial, because both trials lacked appropriate and standardised protocols for taking radiographs. Although it is not likely that this influenced the results much [9], it is necessary to reproduce them in a study with

well-standardised protocols. These two long-term trials both looked at the effect of GlcN-S on knee OA, no trial has been or is being performed yet that looks at its effect on hip OA.

Based on the above, we designed a long-term trial to answer our main question: Does glucosamine sulphate favourably modify progression of osteoarthritis? Because there still is uncertainty about the symptom-modifying properties of GlcN-S we will also try to answer three secondary questions: Does GlcN-S have the same effects in all stages of OA? What is the long-term cost-effectiveness of addition of GlcN-S to usual care? And, does GlcN-S prevent the onset or progression of OA in the contralateral hip joint? Additional to these clinical questions, the data will be used to look at changes on cell-level caused by GlcN-S to learn more about its possible mechanisms of action. All results derived from this trial will be published using our International Standardised Randomised Controlled Trial Number (ISRCTN).

In this article we will present the detailed protocol of the trial. This trial is ongoing; at the moment all patients are included and have passed the first 9 months of the follow-up period.

METHODS/DESIGN

Study Design

This study is a randomised, blinded, placebo-controlled trial. All actors in this trial, who may cause bias, are blinded to treatment allocation: the patient, who is the assessor of the symptomatic outcomes, the researcher, who is the assessor of the objective outcomes, and the caregiver. The analyses will also be performed blind. The study design was approved by the Medical Ethics Committee at the Erasmus MC - university medical centre Rotterdam. All patients gave written informed consent.

Patient selection

General practitioners in the Rotterdam area agreed to search their electronic medical record for patients diagnosed with hip OA and for patients with symptoms associated with hip OA (i.e., persistent hip pain in combination with NSAID use). These patients are contacted by their general practitioner and informed about the trial. For more information, patients can forward their contact details to the researchers. These patients then receive an extensive information folder containing all the information needed to make an informed decision about participation in the study. This folder has been reviewed and approved by the medical ethics committee.

The information folder also contains an informed consent form, which patients need to fill out if they want to participate in the study. Patients who give written informed consent are contacted by phone for a preliminary check of the inclusion and exclusion criteria. People meeting these criteria are invited to the research centre of Erasmus MC for a baseline-measurement, during which the criteria can be checked more precisely.

In- and exclusion criteria

Patients are eligible for inclusion when they meet one of the ACR criteria for hip OA [10]. Patients that have already undergone hip replacement surgery or those on the waiting list for joint replacement are not included in the study. Neither eligible patients with a Kellgren & Lawrence (KL) score of 4 [11], nor people with renal and/or hepatic disease, diabetes mellitus or a disabling co-morbidity are included. Finally, patients unable to understand Dutch questionnaires are excluded from participation.

Sample size

The sample size was calculated primarily to detect clinically relevant differences in radiological progression of the affected joints between the two groups (treatment and placebo) after two years of follow up. To detect a difference of 0.25 mm in radiological progression (SD 0.5) between the intervention and placebo groups (power 80%, alpha 5%, one-tailed testing) after two years of follow-up, 63 patients with hip osteoarthritis are needed per group. These calculations are based on an average change of 0.33 mm in joint space (SD 0.5) during one year of follow-up of patients with hip osteoarthritis [12].

Fewer patients are needed to detect relevant clinical differences: to detect a difference of 25% in pain (Western Ontario McMaster Universities Osteoarthritis index (WOMAC)) with one-tailed testing, a power of 80%, and alpha 5% (Mean 4.83, SD 2.25 [13]) 55 patients per group are needed. To detect the same difference in function (WOMAC) (mean 4.81, SD 2.18 [13]) 51 patients are needed per group.

As we expect a 20% loss to follow-up, we need to include 150 patients. However, to create options for studying effect-modification by type and severity of osteoarthritis, we oversized this trial to 220 patients (110 in each group).

Intervention

Patients who participate in the trial are randomised to either GlcN-S or a placebo for the duration of two years. To ensure a daily intake of 1500 mg GlcN-S, they are required to take two pills each day. The GlcN-S and placebo pills are identical in taste and appearance and were delivered in identical plastic bottles. This will ensure true blinding of the patients and of the researchers. Blinding of the patients will be tested after two years; if people can guess what sort of pills they were taking, this might have an influence on the subjective measures. This will therefore be taken into account in the analysis of the data. The Department of Nutritional Sciences at Numico Research BV manufactured the pills used in this trial.

Randomisation

Following informed consent and baseline assessments, patients are allocated to the intervention or control group using a blinded randomisation list. The randomisation list contains four different strata and is randomised per block of six numbers. This list was generated with a

Table 1: Outline of the randomisation strata

	Hip radiograph	Knee and hand radiographs	Type
Group 1	KL score < 2	KL score < 2 for hands and knees	mild + localised
Group 2	KL score < 2	KL score ≥ 2 for hands and/or knees	mild + generalised
Group 3	KL score ≥ 2	KL score < 2 for hands and knees	moderate/severe + localised
Group 4	KL score ≤ 2	KL score ≥ 2 for hands and/or knees	moderate/severe + generalised

computer by an independent researcher. This researcher also handled labelling the pill-bottles with the randomisation numbers. The researchers involved in this project received all bottles after they were labelled, ensuring blinding to treatment allocation. The randomisation list with the key to treatment allocation will be kept in a safe until the end of the trial. To be able to perform the analyses blinded, the allocation to treatment A and treatment B will be provided, but not the key to A and B.

People are assigned to one of the four different strata on the basis of the Kellgren-Lawrence score of the hips, knees and hands. A researcher (RMR) will score all the radiographs according to the Kellgren-Lawrence score. The outline of the four different strata is given in table 1. Once the correct stratum is established at baseline, the patient is given the subsequent unique four-digit randomisation number from his/her stratum on the randomisation list. This number is used for labelling study materials and data. By stratifying, patients are optimally distributed to GlcN-S and placebo in the different strata, which makes comparing people with mild OA to people with moderate-severe OA, and comparing people with local OA to people with generalised OA possible. In this way, we will be able to study whether effect of treatment depends on severity or localisation of OA.

Measurements

Data for the primary and secondary outcome measures are being collected at different time-points throughout the trial. An overview of the timing of the measurements and the outline of the primary and secondary outcome measures is given in table 2.

In brief, the trial starts for every patient with a baseline assessment at the research centre. At the end of this assessment, patients receive a supply of GlcN-S or placebo sufficient for seven months. After the baseline assessment, patients will receive a questionnaire every three months, which has to be returned to the researchers, except from those at 6, 12 and 18 months after baseline, which will be collected by the researchers during a home visit. After two years, patients return to the research centre for the final assessment, which marks the end of the trial. The collection of the outcome measures is described in the following sections.

Radiographs

Radiographs are taken during the baseline assessment and during the final assessment two years later.

Table 2: Timing of measurements and outline of primary and secondary outcome measures

	0m B.A.	3m Q	6m Visit	9m Q	12m Visit	15m Q	18m Visit	21m Q	24m F.A.
Primary outcome measures									
Joint space width	x								x
Pain score (WOMAC)	x								x
Function score (WOMAC)	x								x
Secondary outcome measures									
Subchondral bone quality	x								x
Stiffness score (WOMAC)	x	x	x	x	x	x	x	x	x
Quality of life (EuroQol EQ-5D)	x	x	x	x	x	x	x	x	x
Medical consumption	x	x	x	x	x	x	x	x	x
Side effects		x	x	x	x	x	x	x	x
CTX-II	x		x		x		x		x
Possible confounders/ Effect modifiers									
Type of OA (localised – generalised)	x								
Radiological severity	x								x
Joint function	x								x
Age	x								
Gender	x								
Activity level	x	x	x	x	x	x	x	x	x
Co-interventions	x	x	x	x	x	x	x	x	x
Compliance (BMQ)		x	x	x	x	x	x	x	x
Compliance (pill count)			x		x		x		x

Note: 0m: 0 month of follow up, 3m: 3 months of follow up etc. B.A.: baseline assessment. Visit: 6 monthly visit. F.A.: final assessment

At baseline, three anteroposterior (AP) radiographs are taken, one of the pelvis, one of both knees, and one of both hands. All radiographs are used to establish what stratum the subject belongs to (table 1). The radiographs of hands and knees will not be used to determine outcome measures and will therefore not be repeated at follow up. As follows from table 1, people with knee and/or hand OA are stratified to one of the 'generalised OA' groups (2 or 4).

A highly standardised protocol is used to make the weight-bearing, AP pelvic radiographs at baseline and follow-up, allowing for a correct measurement of our primary outcome variable: joint space narrowing. The patients' feet are positioned alongside a frame, which was designed to ensure 15° internal rotation of the hips. A second frame (no internal rotation) is available for patients with severe mobility restrictions of the hips. The frame used during the baseline radiograph of a patient will also be used two years later for his/her follow up radiograph. Patients are asked to stand upright. If present, flexion in hips or knees is recorded. Protocol for the pelvic X-ray further states that focus-to-film distance should be 130 cm and that the X-ray beam should be centred on the superior aspect of the pubic symphysis. The X-rays are digitised.

The X-rays from baseline and final assessment will be analysed side by side. The minimal joint space width (JSW) will be identified from the baseline X-ray by assessing four different points: medial, axial, superior and lateral [14]. The researcher will also identify a point that appears to be the minimal JSW. From these five points the actual minimal JSW will be determined. This point will be used to measure changes in joint space width over the two-year follow up period.

DEXA-scan

During the baseline and final assessment, a Dual Energy X-ray Absorptiometry (DEXA) scan will be used to make scans of the pelvis. A frame similar to the one used for the radiograph of the hips is also used to make the DEXA-scan, ensuring 15° internal rotation of the hips. The scan will be used to study quantitative changes in subchondral bone density both of the affected joint and of the contra-lateral joint. Subchondral bone-density alterations might indicate osteoarthritic progression. This long-term trial can be helpful to determine whether pre-clinical OA can be recognised from a DEXA-scan. And, if so, whether GlcN-S prevents the onset or progression of OA in the pre-clinical stage.

Physical examination

A physical examination is carried out at baseline and is repeated during the final assessment. At baseline, this test is first of all used to check part of the inclusion criteria. Various tests are also carried out to check for co-existing musculoskeletal disorders. Findings from the physical examination will be used as baseline characteristics, and to register clinical signs and joint function after two years of follow up. Joint function is established by assessing pain due to joint motion, and by measuring limitation of joint motion with a two-arm goniometer.

Questionnaires

Throughout the study, patients will fill out a total of nine questionnaires. The first during the baseline assessment, followed by a questionnaire every three months in the following two years (including the last one during the final assessment).

The baseline questionnaire is used to measure different patient characteristics (age, gender, race, social status, Body Mass Index (BMI)), disease related characteristics (localisation of symptoms, duration of symptoms, family history) and co-morbidities. Of these characteristics, BMI and co-morbidities will be monitored throughout the trial.

Three validated instruments are used in all nine questionnaires: the WOMAC questionnaire will be used to establish severity of clinical status. It contains subscales for pain, stiffness and function. The WOMAC questionnaire is extensively validated and recommended for clinical assessment in osteoarthritis trials by the WHO [15]. The EuroQol (EQ-5D) will be used to measure quality of life, because of the usefulness of this scale in cost-effectiveness analysis [16,17]. The cost-effectiveness analysis will also be based on employment status, sick leave, changes in

work-tasks or other work-related adjustments, and on medical consumption. The SQUASH questionnaire is used to measure load level in work and sports [18].

In the eight follow-up questionnaires, patients will be asked to answer questions about alterations in their symptoms (i.e., whether they improved or deteriorated), which will be measured with a 7-point Likert scale. Also, compliance to treatment is measured with the Brief Medication Questionnaire (BMQ) [19].

Laboratory assessments

At baseline, two samples of blood are collected. The first to measure the erythrocyte sedimentation rate (ESR), which is used for the inclusion criteria (ACR-criteria). The second sample is stored at –20°C to create options for future DNA-research, for which patients gave separate written informed consent.

Throughout the study, we will collect samples of second-morning void urine of all patients. In urine a marker of cartilage degradation can be found, called CTX-II. In the Rotterdam study [20] this marker was found to be predictive of radiological progression of hip and knee OA. It may therefore be used to assess the effect of treatment on the progression of OA. Urine samples will be collected at baseline and once every six months during follow up. At the end of the study a total of five samples will be available from every patient. These urine samples are stored at –80°C. If promising new markers are discovered during the course of the study, these can also be included in the analysis.

Half-yearly visits

Every six months one of the researchers will visit the patients at home. The main reason for this visit is to provide the patient with new pills (sufficient for seven months). To be able to calculate compliance to treatment, the pills remaining of the previous supply will be collected. The amount of remaining pills combined with the score on the compliance questionnaire (BMQ) will give a good indication of the actual amount of pills the patient has been taking. Finally, a sample of second-morning void urine on the day of the visit will be collected.

Analyses

The researchers will be aware of allocation to treatment A or B at the time of the statistical analyses, but will not know which group received GlcN-S and which group received the placebo. All analyses will take place after the trial has finished, no intermediate analyses will be performed.

Success of randomisation and normality of outcome measures will be checked before actual analyses are done. Differences in the primary outcome measures JSN and WOMAC (pain and function) between the intervention and placebo group will be analysed on the basis of the 'intention to treat' principle using linear regression models. Additionally a per-protocol analysis will be done. When it turns out that randomisation was (partially) unsuccessful, we will adjust

for differences in prognosis. Using baseline characteristics, we can identify factors that influence outcome of the study. Factors that change the outcome with 10% will be regarded as confounders and will therefore be added to the regression-model.

A cost-effectiveness analysis will be performed from a social and a patient perspective, looking at differences in direct and indirect health care cost between the two groups (GlcN-S and placebo). If the trial does not show a difference in disease parameters (WOMAC) and quality of life (EuroQol) between the GlcN-S and the placebo group, the analysis will be reduced to a cost minimisation analysis. This form of analysis evaluates the efficacy of treatment based solely on direct and indirect costs. If the study does find a positive difference in disease parameter and/or quality of life a cost-effectiveness ratio can be determined with on the one hand the costs and savings and on the other hand the disease-specific parameters and also quality of life.

Current status

A total of 40 GP's were found willing to participate in the study. They sent a total of 600 letters to inform possible eligible patients of the study. We received 417 requests for additional information and thus sent an equal amount of information folders. Of these 417 people 250 returned a written informed consent. Eventually 222 people entered the study. Of the 28 people that did not enter the study, most did not meet the inclusion criteria and a few people changed their mind and withdrew their informed consent before randomisation.

We started including patients at the end of September 2003 and the last patient was included on March 15th of 2004. This means the study will run until March 2006. The first results will be available around September 2006.

REFERENCES

1. Lequesne M, Brandt K, Bellamy N, Moskowitz R, Menkes CJ, Pelletier JP, *et al.* Guidelines for testing slow acting drugs in osteoarthritis. J Rheumatol Suppl 1994;41:65-71; discussion 2-3.
2. Towheed TE, Judd MJ, Hochberg MC, Wells G. Acetaminophen for osteoarthritis. Cochrane Database Syst Rev 2003:CD004257.
3. McAlindon TE, LaValley MP, Gulin JP, Felson DT. Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis. Jama 2000;283:1469-75.
4. Towheed TE, Anastassiades TP, Shea B, Houpt J, Welch V, Hochberg MC. Glucosamine therapy for treating osteoarthritis. Cochrane Database Syst Rev 2001:CD002946.
5. McAlindon T, Formica M, LaValley M, Lehmer M, Kabbara K. Effectiveness of glucosamine for symptoms of knee osteoarthritis: results from an internet-based randomized double-blind controlled trial. Am J Med 2004;117:643-9.
6. Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, *et al.* Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357:251-6.
7. Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC. Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 2002;162:2113-23.
8. Hochberg MC, Altman RD, Brandt KD, Moskowitz RW. Design and conduct of clinical trials in osteoarthritis: preliminary recommendations from a task force of the Osteoarthritis Research Society. J Rheumatol 1997;24:792-4.
9. Pavelka K, Bruyere O, Rovati LC, Olejarova M, Giacovelli G, Reginster JY. Relief in mild-to-moderate pain is not a confounder in joint space narrowing assessment of full extension knee radiographs in recent osteoarthritis structure-modifying drug trials. Osteoarthritis Cartilage 2003;11:730-7.
10. Altman R, Alarcon G, Appelrouth D, Bloch D, Borenstein D, Brandt K, *et al.* The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. Arthritis Rheum 1991;34:505-14.
11. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494-502.
12. Dougados M, Gueguen A, Nguyen M, Berdah L, Lequesne M, Mazieres B, *et al.* Radiological progression of hip osteoarthritis: definition, risk factors and correlations with clinical status. Ann Rheum Dis 1996;55:356-62.
13. Angst F, Aeschlimann A, Steiner W, Stucki G. Responsiveness of the WOMAC osteoarthritis index as compared with the SF-36 in patients with osteoarthritis of the legs undergoing a comprehensive rehabilitation intervention. Ann Rheum Dis 2001;60:834-40.
14. Reijman M, Hazes JM, Pols HA, Bernsen RM, Koes BW, Bierma-Zeinstra SM. Validity and reliability of three definitions of hip osteoarthritis: cross sectional and longitudinal approach. Ann Rheum Dis 2004;63:1427-33.
15. Dieppe PA. Recommended methodology for assessing the progression of osteoarthritis of the hip and knee joints. Osteoarthritis Cartilage 1995;3:73-7.
16. van Agt HM, Essink-Bot ML, Krabbe PF, Bonsel GJ. Test-retest reliability of health state valuations collected with the EuroQol questionnaire. Soc Sci Med 1994;39:1537-44.
17. Dolan P. Modeling valuations for EuroQol health states. Med Care 1997;35:1095-108.
18. Wendel-Vos GC, Schuit AJ, Saris WH, Kromhout D. Reproducibility and relative validity of the short questionnaire to assess health-enhancing physical activity. J Clin Epidemiol 2003;56:1163-9.
19. Svarstad BL, Chewning BA, Sleath BL, Claesson C. The brief medication questionnaire: a tool for screening patient adherence and barriers to adherence. Patient Educ Couns 1999;37:113-24.
20. Reijman M, Hazes JM, Bierma-Zeinstra SM, Koes BW, Christgau S, Christiansen C, *et al.* A new marker for osteoarthritis: cross-sectional and longitudinal approach. Arthritis Rheum 2004;50:2471-8.

This thesis was financially supported by:

Nederlandse Orthopaedische Vereniging
Dutch Arthritis Association
Stichting Anna Fonds
J.E. Jurriaanse Stichting
Biomet Nederland B.V.
Link Nederland
GlaxoSmithKline B.V.
Arthrex Nederland B.V.
Oudshoorn Chirurgische Techniek B.V.
Synthes B.V. Netherlands
Bauerfeind Benelux B.V.
Heraeus Medical
LIVIT Orthopedie B.V.
Penders Voetzorg
Mölnlycke Health Care B.V.
Van Wijk Voet & Zorg