Advanced Glycation Endproducts in the Development of Osteoarthritis: Cartilage Biochemistry and Biomechanics

Nicole Verzijl

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Advanced Glycation Endproducts in the Development of Osteoarthritis: Cartilage Biochemistry and Biomechanics

Niet-Enzymatische Glycering
in het Ontstaan van Artrose:
Kraakbeen Biochemie en Biomechanica
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op dinsdag 19 juni 2001 des middags te 13.30 uur

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geboren op 20 oktober 1971, te Maasdam

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STELLINGEN

behorende bij het proefschrift

Advanced Glycation Endproducts in the Development of Osteoarthritis: Cartilage Biochemistry and Biomechanics

1. De snelheid van accumulatie van niet-enzymatische glyceringsproducten in een eiwit wordt voor een belangrijk deel bepaald door de vervangingssnelheid van het betreffende eiwit. *Dit proefschrift.*

2. Cross-linking van kraakbeen collageen door niet-enzymatische glyceringsproducten leidt tot het stijver worden van het collageennetwerk. *Dit proefschrift.*

3. Mensen met beginnende knie artrose vormen sneller niet-enzymatische glyceringsproducten dan gezonde controles. *Dit proefschrift.*

4. Aangezien de vormingssnelheid van pentosidine, een niet-enzymatisch glyceringsproduct, in huidcollageen omgekeerd evenredig is met de maximale levensduur van een soort, mogen wij ons gelukkig prijzen slechte pentosidine-vormers te zijn. *Proc. Natl. Acad. Sci. U.S.A. 1996; 93: 485-490.*

5. Makkelijkere publicatie van 'negatieve' resultaten van wetenschappelijk onderzoek zou waarschijnlijk herhaling van vergelijkbare studies door verschillende groepen voorkomen.

6. Aangezien de innovatiekracht van een organisatie sterk afhangt van de creativiteit van haar medewerkers, zou iedere organisatie er naar moeten streven creatieve geesten zoveel mogelijk de ruimte te geven.

7. Het gevoel in het diepe gegooid te zijn wordt vaak gevolgd door het besef op een steile leercurve te zitten.

8. In de discussie over orgaandonatie mag meer aandacht besteed worden aan het feit dat geven en nemen samen horen te gaan.

9. De luidruchtige media-aandacht voor 'stille tochten' doet afbreuk aan de waarde van dergelijke tochten.

10. Voor de illusie met een crème collageen in de huid te kunnen brengen wordt vaak veel betaald.

11. Het restaureren van een oldtimer vraagt zowel de bereidheid (erg) vieze handen te krijgen als de nodige hersengymnastiek.

12. Hedendaagse auto's zijn veelal praktische vervoersmiddelen; oudere auto's zijn het vooral waard om van te genieten.

Nicole Verzijl

Utrecht, 19 juni 2001

ἔπεμψε γὰρ Ἥρα Σφίγγα, ἣ μητρὸς μὲν Ἐχίδνης ἦν πατρὸς
δὲ Τυφῶνος, εἶχε δὲ πρόσωπον μὲν γυναικός, στῆθος δὲ καὶ
βάσιν καὶ οὐρὰν λέοντος καὶ πτέρυγας ὄρνιθος. μαθοῦσα δὲ
αἴνιγμα παρὰ μουσῶν ἐπὶ τὸ Φίκιον ὄρος ἐκαθέζετο, καὶ τοῦτο
προύτεινε Θηβαίοις. ἦν δὲ τὸ αἴνιγμα· τί ἐστιν ὃ μίαν ἔχον
φωνὴν τετράπουν καὶ δίπουν καὶ τρίπουν γίνεται;

(From: Apollodorus, *The Library*, III. v. 8)

Voor mijn ouders

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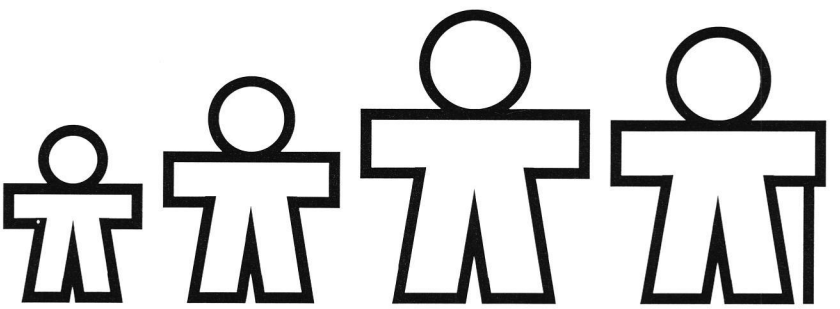


Chapter 1

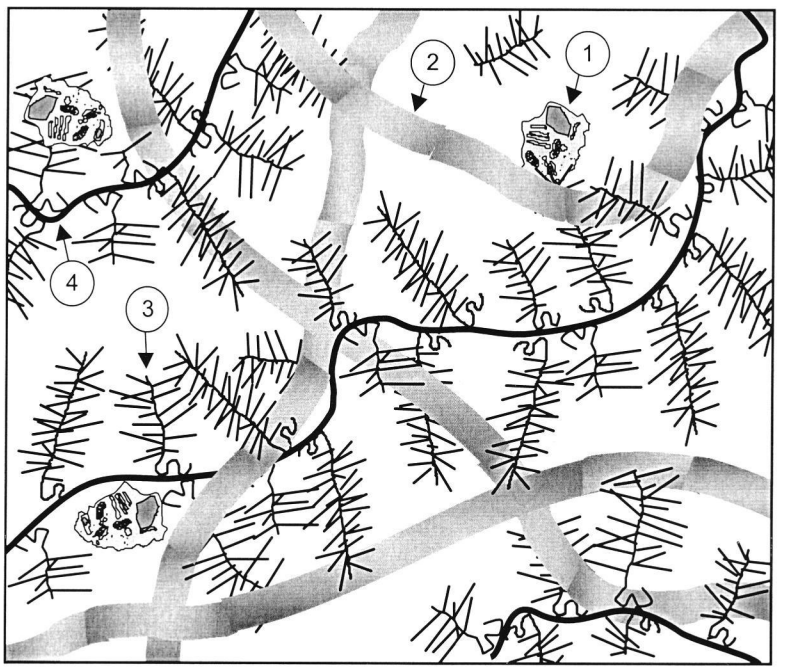
General Introduction

Nicole Verzijl[a,b] and Jeroen DeGroot[a,b]

1. Articular cartilage

Articular cartilage is a highly specialized connective tissue that covers the ends of bones within synovial joints. By means of its unique structure, articular cartilage meets its functional requirements of withstanding compressive and shear forces and transferring these forces to the subchondral bone.[233] In addition, cartilage provides a lubricated surface that facilitates smooth joint movement, even under load. At first sight, cartilage seems to be a simple inert tissue, but upon closer inspection, it appears to contain an elaborate, highly structured extracellular matrix that is deposited and maintained by a relatively small number of highly specialized cells: the chondrocytes (Figure 1). Surprisingly, the major component of the extracellular matrix of articular cartilage is water (70-75% of the weight in adult tissue), while collagen and proteoglycans account for the major portion of the solid material (17-19% and 5-10% of the wet weight, respectively).[62,205] The collagen network defines the form and tensile strength of articular cartilage, while the highly hydrophilic proteoglycans are responsible for the resilience of cartilage (the ability to resume its original shape and texture after a deformation). The proteoglycans are immobilized in the colla-

Figure 1 - Articular cartilage. The main constituents of articular cartilage are depicted. Sparsely present chondrocytes (1) are embedded in a highly hydrated gel of proteoglycans and collagens. The collagen fibrils (2) form a network that gives cartilage its tensile strength. Within the collagen network proteoglycans (3) form large aggregates by binding to hyaluronan chains (4).



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gen network, which results in fixation of a large negative charge within the cartilage matrix. To balance this negative charge, cations are drawn into the tissue thus creating a large osmotic potential. Consequently, water is imbibed into the tissue, generating a large swelling force that, in the unloaded condition, is balanced against the constraining influence of the collagen network. On compressive loading of the joint, water is squeezed out of the cartilage. During unloading, because of the osmotic pressure of the proteoglycans, this water is imbibed again, thus providing the unique resilience of articular cartilage.[62,233] Consequently, the mechanical properties of articular cartilage are highly dependent on the integrity of the collagen network, the retention within the network of a high concentration of proteoglycans, and the capacity of chondrocytes to maintain this extracellular matrix integrity.

1.1. Chondrocytes

Chondrocytes are mesenchymal cells that proliferate and differentiate during development. After growth has ceased (in adult cartilage) there is no detectable cell proliferation. In adult human cartilage, the chondrocytes occupy 1-5% of the tissue volume, the remainder being the extracellular matrix.[176,233] Since articular cartilage is avascular, nutrition of chondrocytes depends on diffusion from the synovial fluid, which is facilitated by intermittent loading of the cartilage.[201,233] Consequently, chondrocytes are adapted to exist under oxygen tensions as low as 1% and preferentially use anaerobic metabolism.[233,316] Chondrocytes are the sole cells responsible for the production and assembly of the constituents of the extracellular matrix of cartilage and are thus essential for maintaining integrity of the tissue. To this purpose chondrocytes adjust their behavior in response to external signals. These signals include cytokines,[43,87,179] growth factors,[192] nitric oxide,[318] vitamins,[229,308] and expectedly matrix degradation products.[145,266] In addition, changes in mechanical and hydrostatic loading patterns influence the functioning of the chondrocytes.[232,314]

1.2. Extracellular matrix

1.2.1. Collagens

Collagens consist of three polypeptide chains (α-chains), each possessing the characteristic tripeptide sequence glycine-X-Y in which proline most often occupies the X position and hydroxyproline the Y position.[211] Three α-chains tightly entwine into a triple helix for which the presence of glycine on every third position is a prerequisite (only glycine is small enough to fit inside the helix).[211] In articular cartilage, approximately 95% of the collagen is type II collagen. Its polymers are the fibrils that form the basic cohesive framework of the tissue, which physically entraps aggregating proteoglycans.[176]

Collagen type II belongs to the fibril forming collagens (class I or interstitial collagens) and is a homotrimer of α1(II) chains.[348] The collagen biosynthesis involves several unique posttranslational modifications (Figure 2). After translation of the procollagen α-chain, specific proline and lysine residues are hydroxylated by prolyl and lysyl hydroxylase, respectively.[172] Hydroxyproline is needed for the formation and stabilization of the triple helix by hydrogen bonds with proline residues. Hydroxylysine residues serve as sites for enzymatically regulated O-linked glycosylation and cross-linking of collagen.[171,272] The function of collagen glycosylation is not yet clear but it has been postulated to play a role in determining fibril diameter[56,365] and in decreasing the protease susceptibility of collagen and thus contributing to collagen stability.[365] Glycosylation levels in type II collagen are the highest found in interstitial collagens, reaching levels of ~20 residues per collagen molecule in human articular cartilage (*i.e.* ~45 percent of the hydroxylysine residues is modified).[26] Hydroxylation and glycosylation cease when the α-chains entwine to form the procollagen triple helix which is secreted and processed for incorporation into the extracellular network.

In the extracellular space, the terminal propeptide regions are released by procollagen peptidases.[76,187,273] This results in a de-

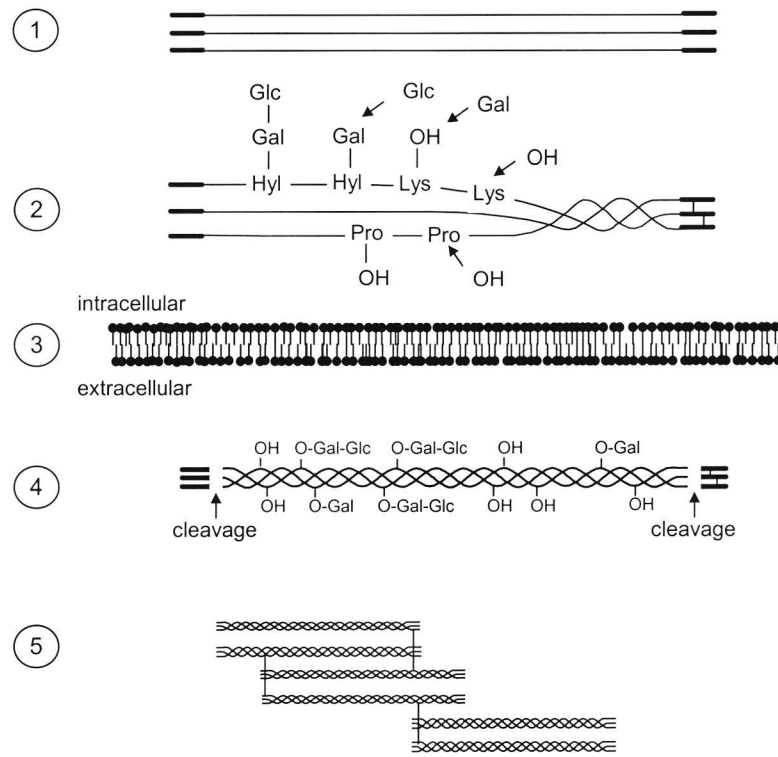


FIGURE 2 - Collagen synthesis. Collagen is synthesized as propeptide-containing α-chains (1) that are post-translationally modified by hydroxylation of lysyl and prolyl residues and by glycosylation of hydroxylysyl residues (2). These modifications cease when three α-chains entwine to form a collagen triple helix (2). Triple helical collagen molecules are secreted from the cell (3) and the propeptides are cleaved off extracellularly (4). Subsequently, collagen molecules spontaneously assemble into fibrils with quarter-staggered overlap of the individual triple helices. Finally, the fibrils are stabilized by formation of intermolecular pyridinoline crosslinks (5).

crease in solubility, forcing the triple helical collagen molecules to self-assemble into fibrils. In the collagen fibrils, the individual molecules are orientated in parallel with quarter-staggered overlap. This highly organized collagen arrangement results in the characteristic banded pattern when visualized by electron microscopy.[94] After fibril formation, intermolecular pyridinoline cross-links are formed. This enzymatic cross-linking process is initiated by lysyl oxidase[306] and results in the formation of trifunctional cross-links that play an important role in determining the ultimate structural integrity of the collagen network. In cartilage, hydroxylysylpyridinoline (HP; derived from three hydroxylysine residues) is found in 30-50 fold higher levels than lysylpyridinoline (LP; derived from two hydroxylysine residues and a lysine residue at the triple-helical cross-linking site).[25,96]

The minor collagens present in cartilage are mainly type IX (1-2%) and XI (3-4%) collagen.[95] Type IX collagen is present on the surface of the type II collagen fibrils and may function as an interfibrillar connector that not only facilitates the formation and organization of the three-dimensional collagen network, but also enhances its stability.[95,124,150] In addition, type IX collagen provides a covalent interface between the surface of the type II collagen fibril and the interfibrillar proteoglycans.[95] Type XI collagen is present within the interior of the fibrils

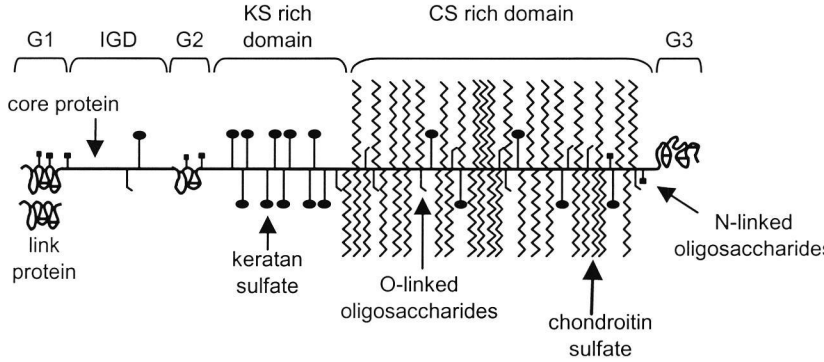


FIGURE 3 - Schematic representation of the structure of an aggrecan monomer and link protein. Two of the globular domains (G1 and G2) are separated by the interglobular domain (IGD). Between G2 and the third globular domain (G3) a keratan sulfate (KS) rich domain and a chondroitin sulfate (CS) rich domain can be identified in the core protein. Furthermore, the core protein contains O- and N-linked oligosaccharides. The link protein stabalizes the interaction of the G1 domain with hyaluronan.

and is suggested to help in fibril formation and determine the final diameter of the fibril.[176,218] In addition, type VI collagen (<2% of the total collagen amount) forms a separate microfibrillar network in the pericellular matrix that acts as a bridge between the chondrocyte and the interterritorial matrix.[268,274]

1.2.2. Proteoglycans

The predominant proteoglycan in articular cartilage is aggrecan, comprising ± 90% of the cartilage proteoglycans. Aggrecan is a large, highly polyanionic macromolecule that forms large aggregates of ± 100 MDa. Aggrecan consists of a central core protein with a multi-domain structure, each domain endowing the molecule with specific characteristics (Figure 3).[133] The N-terminal region contains two globular domains (G1 and G2) separated by an interglobular domain (IGD) that contains several proteinase cleavage sites. The G1 domain contains an immunoglobulin fold and two proteoglycan tandem repeat structures by which it interacts with hyaluronan (a long chain glycosaminoglycan).[107] One hyaluronan chain can bind up to 200 aggrecan monomers thus forming large proteoglycan aggregates. The aggrecan-hyaluronan interaction is stabilized by link protein, a 40 kDa globular protein with high homology to the G1 domain of aggrecan, in a 1:1 molar ratio (aggrecan:link protein).[131,176] At the C-terminal end of the aggrecan core protein a third globular domain is present (G3) that is highly conserved between different species and mediates interactions with other matrix constituents.[249] In between the G2 and G3 domains, the highly negatively charged keratan sulfate (KS) and chondroitin sulfate (CS) glycosaminoglycans are attached to the core protein. This results in the characteristic brush-like structure. Each core protein contains ± 50 KS chains, attached to the KS-rich region and ± 100 CS chains, attached to the CS-rich region. In addition, KS is found in the IGD domain.[133] The presence of these KS chains in the IGD is required for aggrecanase cleavage of the core protein and is thus involved in the stability of the aggrecan molecule.[270]

The small leucine-rich proteoglycans (such as decorin, fibromodulin, biglycan, lumican and epiphycan) present a small fraction of the total mass of proteoglycans within the cartilage extracellular matrix. Being small sized they represent molar contents similar to or exceeding that of aggrecan.[267,312] Decorin and fibromodulin interact with collagen type II and are suggested to play a role in the formation and maintenance

of the collagen fibrils.[124,183] Biglycan is found primarily in the pericellular matrix and may interact with type VI collagen.[320] The detailed localization and possible functional role of lumican and epiphycan still have to be identified.

1.2.3. Noncollagenous, nonproteoglycan matrix constituents

In addition to the collagens and proteoglycans, several minor constituents that are neither collagens nor proteoglycans are present in articular cartilage. The putative functions of these proteins range from involvement in matrix assembly and matrix-cell interactions to modulation of the chondrocyte phenotype. For some of these proteins no functional role has yet been identified.[194,240] As an example, a prominent noncollagenous, nonproteoglycan matrix molecule is COMP (cartilage oligomeric matrix protein) which associates with collagen and is believed to play a role in collagen fibrillogenesis in the pericellular matrix.[282]

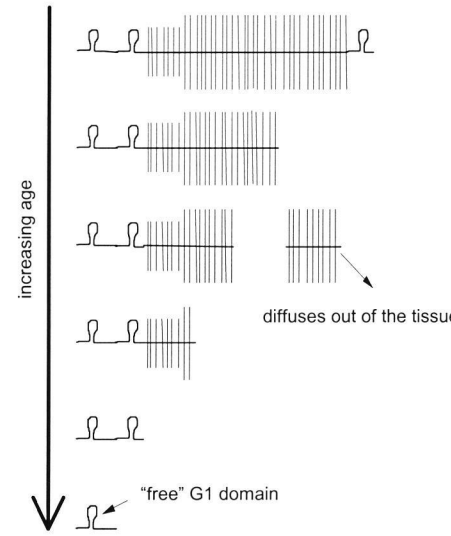


FIGURE 4 - Schematic representation of the turnover of the aggrecan monomer. Degradation of the aggrecan monomer with age results in loss of C-terminal fragments from the tissue and accumulation of the smaller fragments that remain bound to hyaluronan through their G1 domain.

1.3. Extracellular matrix turnover

Cartilage is generally viewed as an inert tissue that merely deteriorates after skeletal maturity has been reached ("wear-and-tear" theory). Indeed, the half-life of type II collagen in healthy human cartilage is estimated to be more than 100 years,[208] implying that collagen turnover is virtually absent in normal adult cartilage. However, since the turnover of all matrix constituents is characterized by the occurrence of both synthesis and degradation, the presence of type II collagen propeptides in adult articular cartilage reveals that remodeling, though slowly, does take place.[242] In addition, matrix metalloproteinase (MMP)-mediated collagen degradation is observed in adult human cartilage.[46] The MMPs form a family of Zn^{2+}- and Ca^{2+}-dependent proteinases of which the expression is transcriptionally regulated by growth factors, hormones and cytokines. The proteolytic activities of the MMPs are tightly controlled by activation from their precursors (proMMPs) and inhibition by endogenous inhibitors such as tissue inhibitors of matrix metalloproteinases (TIMPs) and α2-macroglobulin.[238]

The turnover of the aggrecan fractions in healthy adult human cartilage is much faster than the collagen turnover: its half-life has been estimated to range from 3 to 24 years.[204] Degradation of aggrecan monomers is mediated by MMPs (*e.g.* MMP-8 and -13)[108,109] and by aggrecanases such as aggrecanase-1 and -2.[1,329] Degradation of aggrecan at multiple sites in the core protein results in the formation of aggrecan molecules of different length.[330] C-terminal fragments diffuse out of the tissue whereas the N-terminal fragments, independent of their length, remain bound to the hyaluronan via their G1 domain.[131,204] Effectively, this results in increased heterogeneity in size of the aggrecan monomers with increasing age, with the smallest fragments having the highest residence time in the matrix (Figure 4).[204] Synthesis of proteoglycans in cartilage is seen throughout the adult tissue and is coordinated with proteoglycan degradation by feedback to the chondrocyte to ensure that

the extracellular matrix content of proteoglycans remains constant.[131]

The turnover of the hyaluronan chains in articular cartilage is less well studied. Coordinated synthesis of hyaluronan and aggrecan has been shown for normal bovine cartilage suggesting similar turnover rates for these molecules.[230] Degradation of hyaluronan is accomplished via receptor-mediated endocytosis and subsequent intracellular degradation.[48]

1.4. Cell - matrix interactions

Cartilage homeostasis is, in addition to soluble factors such as cytokines, dependent on the interactions of the chondrocytes with the extracellular matrix. Differences in composition or structure need to be reflected in an adaptation of cellular behavior. When taken out of the extracellular matrix, chondrocytes rapidly dedifferentiate and start producing inappropriate, non-cartilage-specific proteins.[44,45,346] The interactions between the extracellular matrix and the chondrocytes are mediated by several classes of highly specific transmembrane receptors. The type II collagen is connected to the chondrocytes via integrin receptors and anchorin CII (=annexin V). In addition, the integrins provide chondrocytes with binding sites for fibronectin and other matrix constituents.[191] Integrin-mediated chondrocyte-matrix interactions are essential in chondrocyte differentiation, survival, and response to mechanical stimuli.[138,143] Hyaluronan-binding by chondrocytes is mediated by the CD44 receptor, thereby providing a link between the proteoglycans and the cells.[15,132] The binding of hyaluronan to CD44 is essential in the formation and retention of the pericellular matrix.[153,173] Loss of this interaction results in depletion of aggrecan from the matrix.[70]

In addition, chondrocytes respond to specific degradation products of matrix constituents. Since these products are derived from the extracellular matrix, they can be considered markers that communicate the health status of the surrounding extracellular matrix to the chondrocyte.[144]

1.5. Cartilage mechanical properties

During weight-bearing and joint motion, cartilage experiences substantial compressive stresses as well as tensile and shear stresses. The mechanical properties of the tissue that are needed to withstand the large forces depend on the structure, composition, and organization of its extracellular matrix components, with different matrix constituents contributing differently to the various properties.[298] The strength of cartilage when exposed to tensile stresses, depends to a large extent on the intrinsic stiffness of the collagen fibers and the collagen content, and less on the proteoglycan content.[159,162,283] In contrast, resistance of the matrix to compressive loading in confined compression and indentation tests,[161] which results in fluid exudation from the tissue and fluid redistribution within the tissue, is largely determined by proteoglycans.[160] Nevertheless, tensile forces in the collagen network have recently been suggested also to play an important role in determining tissue behavior in confined compression.[167] Furthermore, in unconfined compression the instantaneous deformation of cartilage is mainly controlled by the collagen network, while proteoglycans play an indirect role by modulating the tensile stresses in the collagen network.[224] Similar findings are reported for the resistance of cartilage to deformation in shear: the collagen fibers appear to be chiefly responsible for this cartilage property, while the proteoglycans assist by generating a swelling pressure that permits the matrix to more effectively resist shear.[298,367]

These relations between cartilage mechanical properties and matrix constituents indicate both how the different matrix components contribute to the mechanical properties of the tissue and, alternatively, how - using mechanical tests - the properties of the cartilage matrix components can be studied. As an alternative to mechanical tests, the tensile properties of the collagen network can also be measured using the "osmotic stress technique",[35] which uses the application of osmotic stress to cartilage by means of calibrated solutions of polyethyl-

ene glycol. The tensile stress exerted by the collagen network, P_c, can be calculated from the 'balance of forces' at equilibrium hydration. At equilibrium hydration, P_c together with the externally applied osmotic stress, π_{PEG}, both of which tend to squeeze water out of the tissue, are balanced by the osmotic pressure of the cartilage proteoglycans, π_{PG} (*i.e.* $P_c + \pi_{PEG} = \pi_{PG}$).

2. CARTILAGE AGING

During aging of articular cartilage, both the extracellular matrix and the chondrocytes are susceptible to change. Many age-related changes in the structure and composition of human articular cartilage aggrecan have been described. With increasing age, the aggrecan KS content steadily increases while the CS content remains constant, which results in an increase in the proportion of KS to CS.[205] Furthermore, an increase in CS 6-sulfation relative to 4-sulfation and a relative increase in the protein content of aggrecan have been demonstrated.[205,262] Some of these changes may be explained by the age-related decrease in the hydrodynamic size of the aggrecan monomers, which is the result of C-terminal degradation fragments diffusing out of the tissue, while the remaining hyaluronan-bound N-terminal fragments accumulate with age (Figure 4).[38,131,204]

Despite an increase in hyaluronan concentration in articular cartilage with age,[142] the rate of incorporation of aggrecan monomers into aggregates is slower in mature cartilage than in tissue from young individuals,[39] which may contribute to the decreased capacity to assemble large aggregates with increasing age.[339] In addition, the ratio of newly synthesized link protein to aggrecan decreases with age, which may result in a higher proportion of link protein-deficient, less stable aggregates with advancing age.[50]

The net effect of all combined age-related changes in aggrecan composition is an increase in the fixed charge density (FCD) of human articular cartilage with age, which results in an age-related increase in osmotic swelling pressure and thus in the resistance of the tissue to fluid loss.[122] Nevertheless, the water content of human articular cartilage decreases somewhat with age.[281,338] The water content of unloaded cartilage is the result of the balance between the tendency of the highly hydrophilic proteoglycans to imbibe water and the tensile stresses in the collagen fiber network that entraps the proteoglycans. Thus, the age-related decrease in water content of human articular cartilage, in combination with an increase in the osmotic swelling pressure of the proteoglycans, indicates that the stiffness of the collagen network must increase with age.[122]

Indeed, Basser *et al.*[35] showed an age-related increase in the tensile stiffness of the articular cartilage collagen network. This increased stiffness cannot be explained by a change in enzymatic collagen cross-linking (both HP and LP concentrations in cartilage collagen are constant with age),[25,96] nor by the slight age-related decrease in collagen content.[338] As far as the relative contribution of different collagen types is concerned, a decrease in the content of collagen types IX and XI is observed with advancing age, which may modify the properties of cartilage but is not expected to increase the stiffness of the collagen network.[95,336] Yet, cross-links derived from the process of nonenzymatic glycation increase with age in cartilage and may explain the age-related increase in collagen stiffness (see paragraph 4).[25,331]

Age-related changes have also been observed in the structure or content of some of the minor cartilage matrix constituents. For example, the contents of lumican and some of the noncollagenous, nonproteolgycan matrix molecules in human articular cartilage increase with age.[121,194,217] Reports on the effect of aging on the amounts of decorin, biglycan, and fibromodulin are less consistent: both increased and decreased levels of biglycan and decorin have been reported.[284,343] Furthermore, the structure of the KS chains that are attached to fibromodulin change with age.[183] The possible effects of these age-related changes in the amount or composition of the minor cartilage molecules on tissue function are not yet known.

In addition to changes in the extracellular matrix with increasing age, the number and behavior of the chondrocytes is subjected to age-related changes. The chondrocyte number is reported to decline with age in femoral head cartilage,[342] whereas knee and shoulder cartilage have been shown to contain stable cell numbers with increasing age.[215,315] Cell functions are generally believed to slightly decline with advancing age[63] but undeniable evidence to support this is lacking. Nevertheless, some age-related changes in chondrocyte function have been described, such as a decrease in link protein mRNA expression[49] and a change in sulfation of the nonreducing terminal mono- and disaccharides on aggrecan CS.[262]

3. OSTEOARTHRITIS

Osteoarthritis (OA), or cartilage degeneration, is a widely prevalent chronic disabling condition. Clinically, OA is characterized by joint pain, tenderness, limitations of movement, crepitus, occasional effusion, and local inflammation secondary to the disease process.[259] The main pathological characteristic of OA is progressive destruction of the articular cartilage. In many cases, subchondral cysts and sclerosis, osteophyte formation and mild synovial inflammation are observed.[149] Ample evidence suggests a multifactorial etiology of OA by combinations of biomechanical, biochemical and genetic factors.[103,104] However, the initial event that triggers the pathological process is unclear and it is still being debated whether the initial changes occur in the cartilage, the subchondral bone or even in the synovium.[85] Risk factors that are identified for OA include general factors that contribute to the overall sensitivity to develop OA (e.g. age, obesity, gender, and genetic predisposition) and local biomechanical factors that determine the location and severity of OA (e.g. joint trauma, anatomical deformities, and occupation).[77,102,105,181] By far the single greatest risk factor for the development of OA is age.[78,105,333] As an example, the prevalence of radiological OA in the female knee joint increases from 12.7% to 40.2% between the ages of 45 and 70 years, while in the distal interphalangeal joint the prevalence at high age is as high as 74.7%.[333] Although age is recognized as the main risk factor for OA, the mechanism by which aging is involved in the etiology of OA remains largely unknown. In general, the disease could be initiated by age-related changes in the shape or stability of the joint, in the structure of the supporting tissues (e.g. bone), and/or in the integrity of the extracellular matrices of cartilage, bone or ligaments.[64] In our view, age-related changes in articular cartilage are likely to be causally involved in the pathogenesis of OA. These age-related changes in articular cartilage predispose to the development of OA but are fundamentally different from the changes that occur in cartilage during the disease process.[122]

One of the earliest features of cartilage degeneration is the increase in swelling of the cartilage.[202] Since the water content of articular cartilage depends on the balance between the swelling pressure of the proteoglycans and the restraining force of the collagen network, swelling of the tissue indicates loss of integrity of this network.[205] The increase in cartilage swelling is corroborated by a decrease in collagen network stiffness and both are highly correlated with the amount of degraded collagen.[31] Collagen damage early in the process of cartilage degeneration could result from fatigue failure of the collagen network,[111] from mechanical trauma,[117] or from proteolytic degradation of collagen by MMPs, such as MMP-1, -3, -8 or -13.[42,46,300] Presumably in an attempt to repair the damage, type II collagen synthesis is increased in OA cartilage.[242] In addition, synthesis is observed of collagen types that are normally not present in articular cartilage or only at very low levels, such as type III, VI, and X collagen. Due to the different biomechanical properties of these aberrant collagen types, this results in an extracellular matrix that does not meet its functional requirements and repair is therefore ineffective.[5,274,347]

Similar to what is found for collagen, the synthesis and degradation of proteoglycans

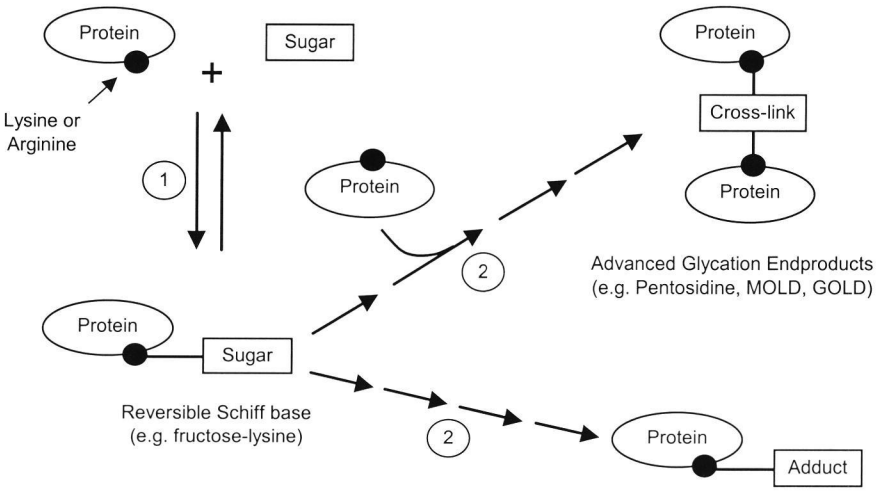


FIGURE 5 - Classical view of AGE formation. Reducing sugars such as glucose or fructose react spontaneously with lysine or arginine residues in proteins. Initially a reversible Schiff base (*e.g.* fructose-lysine) is formed (1). Subsequently, Amadori rearrangement and Maillard browning reactions (2) result in the formation of stable AGEs: some form protein-protein crosslinks, whereas others present protein adducts.

are increased in OA cartilage. The release of proteoglycans is higher from OA cartilage than from normal cartilage,[178] which is consistent with increased MMP and aggrecanase activity in OA cartilage and synovial fluid.[13,42,46,190] Furthermore, proteoglycan synthesis, measured as the rate of sulfate incorporation, is increased two- to four-fold both in the early, preclinical stage and in the late stage of OA.[178,285,322,334] In addition, slightly different proteoglycans are synthesized in OA cartilage; the sulfation pattern of CS chains in OA cartilage proteoglycans is different from normal adult cartilage and resembles that in cartilage at adolescence (15-17 years).[263] The enhanced proteoglycan synthesis does not compensate for the increased release: a net loss of proteoglycans is observed in OA cartilage. This is also due to the decreased retention of newly synthesized proteoglycans in OA cartilage,[178] which may be caused by the damaged collagen network.[31]

4. THE MAILLARD REACTION

During aging, the inescapable chronic exposure of cellular and extracellular matrix proteins to reducing sugars leads to their non-enzymatic modification by these sugars. This process, also known as the Maillard reaction, eventually results in protein browning and cross-linking and is suggested to play a role in the fundamental aging process.[225]

4.1. Chemistry

Nonenzymatic glycation (NEG) is a common posttranslational modification of proteins by reducing sugars. In the classical view, the glycation reaction is initiated by the nonenzymatic condensation of a sugar with the ε-amino group of a lysine residue (*e.g.* resulting in fructose-lysine; FL) or the ε-guanidino group of an arginine residue to form a reversible Schiff base, that is subsequently stabilized by Amadori rearrangement. This Amadori product undergoes Maillard or browning reactions, that result in the formation of advanced glycation endproducts (AGEs) (Figure 5).[278] More recently, alternative pathways for formation of the Amadori product and AGEs have been proposed, e.g. through metal-catalyzed glucose autoxidation[361] or as the result of lipid peroxidation reactions.[251] This has resulted in the appreciation that there are multiple sources and mechanisms of AGE formation *in vivo*.

Due to the highly diverse reaction pathways leading to AGE formation, AGEs with a variety of chemical structures have been identified. Some AGEs are protein-adducts, whereas others present protein-protein cross-links.[41,60,278] All proteins are prone to AGE formation, but since AGEs cannot be removed from proteins, accumulation of AGEs only occurs in long-lived proteins such as lens crystallins and tissue collagens.[25,90,91,295] A summary of AGEs that have been identified in tissue proteins *in vivo* is provided in Table 1.

The diversity of AGEs that are identified *in vivo* is reflected by the range of methods employed to analyze AGEs. These methods vary from high-performance liquid chromatography (e.g. for pentosidine)[27] and gas chromatography - mass spectrometry (e.g. for CML and CEL)[3,91] to the use of specific antibodies (e.g. for pyrraline).[222] Also, general measures of AGE cross-linking, that do not require sophisticated equipment or antibodies, have successfully been used. These measures include the analysis of protein-bound browning (absorbance at 340 nm) or fluorescence at 370/440 nm,[226,294] and the analysis of the susceptibility of AGE-modified protein to enzymatic digestion,[290] the latter providing to a certain extent a functional measure of cross-linking.

4.2. Effects of AGEs on tissue, cell and protein function

Connective tissue strength is highly dependent upon the amount of cross-links present in collagen.[110,254] The controlled formation of enzymatic cross-links, such as the pyridinolines, enables tissues such as cartilage and bone to balance cross-link levels with functional requirements. This is vividly illustrated in pathologies in which cross-linking is disturbed: e.g. the defective cross-linking in bone of Bruck syndrome patients results in highly brittle bone.[30] In this view, it is clear that the mechanical properties of connective tissues are adversely affected by the accumulation of nonenzymatically formed AGE cross-links. Accumulation of AGEs is correlated with increased tissue stiffness in arteries,[304] lens capsules,[22] skin,[277] tendon,[174] and articular cartilage.[25] Moreover, an increase in AGE levels makes tissues increasingly brittle, and thus more prone to mechanical damage. This effect has been shown for human lens capsules[22] and cortical bone.[66,328] For articular cartilage, a decrease in strength is observed with increasing age,[157,351] coinciding with an increase in AGEs in the tissue.[25] Although no direct correlation between AGE levels and brittleness has been shown, these data suggests that also for articular cartilage the level of

TABLE 1. AGEs that have been identified in human tissue proteins

AGE	Cross-link/adduct	Present in
Pentosidine	cross-link	many tissues e.g. dura mater,[295] skin,[93] articular cartilage,[25] brain,[169] Bruch's membrane[129]
CML (N^{ε}-(carboxymethyl)lysine)	adduct	many tissues e.g. lens,[91] skin,[90] brain[169]
CEL (N^{ε}-(carboxyethyl)lysine)	adduct	lens[3]
Pyrraline	adduct	kidney[222]
Imidazolium salts: - MOLD (methylglyoxal-lysine dimer) - GOLD (glyoxal-lysine dimer)	cross-link	lens and skin[57]
Vesperlysine A	cross-link	lens[323]
Argpyrimidine	adduct	cornea,[299] diabetic kidney[255]
Imidazolones	adduct	diabetic aorta and kidney,[243] diabetic retina[128]
NFC-1 (non-fluorescent cross-link 1)	cross-link	diabetic skin,[21] aorta[304]

AGEs determines its resistance to mechanical trauma or fatigue failure.[152]

In addition to affecting the mechanical properties of tissues, AGEs also interfere with cellular processes. It has been suggested that many of the effects of matrix glycation are mediated by specific AGE receptors (for review see Ref. 325). AGE-binding receptors include: scavenger receptors types I and II,[286] the receptor for AGE (RAGE),[170,241,287] oligosaccharyl transferase-48 (OST-48, AGE-R1),[188] 80K-H phosphoprotein (AGE-R2),[366] and galectin-3 (AGE-R3).[344] AGE receptors are found on a variety of cell types such as monocytes, macrophages, endothelial cells, pericytes, podocytes, astrocytes and microglia.[325] Cell activation in response to binding of AGE-modified proteins results in activation of key cell signaling molecules, such as NF-κB, and changes in gene expression.[170,288] Depending on the cell type, this can be associated with cell proliferation, oxidative stress, or apoptosis.[155,220,287] In addition, AGE-modified proteins affect the synthesis of matrix components: AGE-modified albumin has been reported to stimulate collagen type IV synthesis in glomerular mesangial cells.[12,75] In contrast, mesangial cells grown in the presence of glycating sugars or on glycated matrix show decreased synthesis of collagen types I and IV.[303] Type I collagen synthesis is also inhibited in endothelial cells and in fibroblast cell lines upon exposure to glycated albumin.[74,253] Furthermore, AGE-modified albumin has been shown to inhibit matrix metalloproteinase-2 mRNA production by mesangial cells while stimulating TIMP-1 expression, which likely results in decreased extracellular matrix proteolysis.[12] In addition to effects mediated by specific AGE receptors, accumulation of AGEs in extracellular matrix proteins also directly interferes with cell-matrix interactions. A decreased adhesion of cells to the extracellular matrix, possibly mediated by glycation of the arginine residue in the RGD (Arg-Gly-Asp) recognition sequence for integrins has been demonstrated for osteosarcoma and fibrosarcoma cells.[127,257]

Besides cell-mediated effects of AGEs on matrix turnover, accumulation of AGEs also directly affects matrix turnover by changing the physical and chemical properties of proteins. AGE-modification of proteins changes their structure such that it may interfere with highly specific enzyme-substrate interactions[10] and thereby prevent proteolysis. In addition, increased inter- and intramolecular cross-linking may diminish the accessibility of the matrix to proteinases or the release of degraded matrix constituents.[20] The latter may result in chondrolysis due to the stimulation of chondrocytes by unremoved matrix degradation products.[144] Alternatively, modification of lysine and arginine residues changes the charge distribution of the protein,[123] thereby influencing its tertiary structure as well as its interactions with other proteins. As a result, the susceptibility of matrix proteins for proteolytic degradation may be changed, which influences tissue turnover. Indeed, a decrease in the susceptibility of extracellular matrix proteins to proteolytic degradation has been reported at elevated AGE levels. Degradation of AGE-modified collagen by matrix metalloproteinases is impaired compared to unmodified collagen.[231] Furthermore, the proportion of pepsin-released skin collagen decreases with age,[290] which coincides with the age-related increase in AGE levels in skin.[93]

5. HYPOTHESIS

Age is undoubtedly the greatest risk factor for OA, but the mechanisms underlying this relationship are largely unknown. Yet, age-related structural changes in articular cartilage are potential factors in increasing the susceptibility of cartilage to develop OA. Since damage to the collagen network is the first sign of cartilage degeneration, the main focus of our research has been on age-related changes in the collagen network in human adult articular cartilage. Prior to the start of this project, it was known that no age-related changes occur in the enzymatic collagen modifications, lysyl hydroxylation and pyridinoline cross-linking, but that the AGE cross-link pentosidine accumulates

with age in human articular cartilage.[25] Furthermore, preliminary data showed increased stiffness of the cartilage collagen network after *in vitro* glycation by ribose.[25] These results led us to hypothesize that the age-related increase in AGE cross-linking in cartilage collagen results in increased stiffness, and subsequently increased brittleness of the collagen network. Increased brittleness may in turn contribute to the age-related failure of cartilage to resist mechanical damage, and thus be a factor that predisposes aged cartilage to damage and, eventually, the development of OA. Consistent with our hypothesis, the stiffness of the collagen network in human articular cartilage increases with age,[35,122] while its resistance to fatigue damage decreases.[157,350] Furthermore, AGEs are expected to impair extracellular matrix turnover by chondrocytes, as has been shown for other cell types such as fibroblasts and mesangial cells.[12,253] This may result in a diminished ability of chondrocytes to maintain or repair matrix integrity and may thereby contribute to the progression of cartilage defects to OA. The combination of increased cartilage brittleness with diminished repair capacity, both due to the age-related accumulation of AGEs, is likely to contribute to the age-related increase in susceptibility to develop OA.

6. OUTLINE OF THIS THESIS

To test our hypothesis that the age-related accumulation of AGEs in articular cartilage predisposes to the development of OA we have used a variety of experimental approaches. The results of these studies are described in two theses. The effects of AGEs on the synthesis and degradation of cartilage extracellular matrix by chondrocytes and the effect of AGE accumulation on the development of OA in a canine *in vivo* model are described in "Advanced glycation endproducts in the development of osteoarthritis: cartilage synthesis and degradation", by Jeroen DeGroot.[82] The present thesis describes the biochemistry of Maillard reaction products in human adult articular cartilage, the effect of protein turnover on the accumulation of AGEs and the effect of AGE cross-linking on the stiffness of the collagen network. Furthermore, the relation between AGE cross-linking of cartilage collagen and the occurrence of cartilage degeneration was studied in a cross-sectional study. The eventual result of age-related changes in the properties of the cartilage collagen network also depends on the network's "starting quality" at adolescence. Therefore, biochemical characteristics of the cartilage collagen network laid down at adolescence were also related to the development of OA at old age.

The work described in the present thesis was aimed at answering the following questions:

Which Maillard reaction products accumulate with age in cartilage collagen and how do their levels compare to those in other long-lived proteins?

The results of our extensive exploration of Maillard reaction products in cartilage collagen are summarized in chapter 2. Sell and Monnier[295] had previously shown that pentosidine levels in tracheal cartilage are relatively high compared to other tissues. Therefore, we set out to determine levels of several well-characterized products of glycation (fructose-lysine) and subsequent AGE formation (CML, CEL, and pentosidine) in cartilage collagen and compared those levels to levels measured in skin collagen and lens proteins. In addition, we determined general measures of glycation and AGE formation, such as AGE fluorescence (at 360/460 nm), browning (absorption at 340 nm), levels of the glycation-sensitive amino acids arginine and (hydroxy-)lysine, and digestibility of cartilage collagen by bacterial collagenase.

What is the influence of the rate of protein turnover on the accumulation of AGEs, and can pentosidine levels be used as a quantitative measure of protein turnover?

It is known from literature that besides sugar concentration, temperature, pH and oxygen tension, the rate of protein turnover

may also play an important role in determining AGE levels in a tissue or protein.[4,166,295] High levels of AGEs accumulate in cartilage compared to other tissues such as skin.[295] Therefore, we measured levels of pentosidine, CML, and CEL in cartilage and skin collagen and compared these AGE levels between the two collagens in relation to their respective rates of turnover (chapter 3). The residence time of these collagens, *i.e.* a measure of protein turnover, was obtained by measuring the percentage D-aspartic acid. This unique measure of protein residence time is based on the relatively fast racemization of aspartic acid from the L-form, in which it is built into proteins, into a racemic mixture of D- and L-forms with advancing age.[135,210]

Thus far, focus has been on AGE accumulation in collagen from human articular cartilage. In chapter 4, we investigated whether pentosidine also accumulates with age in the other major component of the cartilage extracellular matrix, namely aggrecan which has a faster turnover than collagen. Furthermore, pentosidine levels in aggrecan subfractions of known different residence times were used to explore pentosidine levels as a quantitative measure of aggrecan turnover, as can be derived from levels of aspartic acid racemization.[204] Because the OA disease process results in increased aggrecan turnover,[178,182,200] pentosidine levels in aggrecan isolated from OA cartilage were compared to normal cartilage to see whether aggrecan pentosidine levels reflect the increased turnover.

Does AGE cross-linking affect the mechanical properties of the collagen network in articular cartilage?

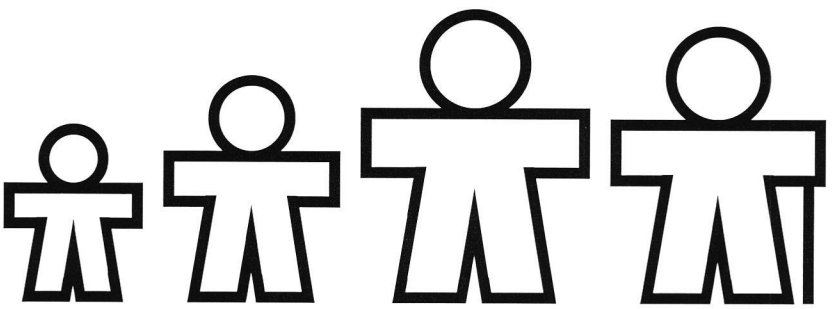
Inasmuch as a substantial number of AGEs are cross-links, we investigated whether AGE cross-linking influences the mechanical properties of the collagen network in articular cartilage. In chapter 5, the effect of *in vitro* glycation of human articular cartilage with threose on the stiffness of the collagen network is described. The collagen network stiffness was assessed by measuring the instantaneous deformation of the tissue after step-loading of a cylindrical cartilage sample in unconfined compression,[224] and confirmed by the osmotic stress technique.[35] Stiffness of the collagen network was related to the level of AGE cross-linking, which was also specifically measured in collagen.

Do AGEs in articular cartilage collagen predispose to the development of OA?

If AGE cross-linking indeed plays a role in increasing the susceptibility of articular cartilage collagen to mechanically induced damage, individuals with a high level of cartilage collagen AGEs may develop OA more often or earlier in life than individuals whose articular cartilage contains less AGEs. To test this hypothesis, AGEs were measured in normal cartilage from joints with focal, preclinical cartilage degeneration at autopsy and compared to cartilage from donors without any sign of degeneration. The results of this comparison are described in chapter 6.

Do properties of the collagen network as it is laid down at adolescence play a role in the development of OA at old age?

The previous chapters have focussed on the age-related accumulation of AGEs in articular cartilage collagen and its influence on the age-related increase in the susceptibility of cartilage for degeneration. However, besides the extent of change with age, the eventual quality of the collagen network will also be determined by its starting quality. In chapter 7, it was investigated whether differences in enzymatic modifications of the cartilage collagen network as it is laid down at adolescence play a role in the development of OA at old age. For this purpose, hydroxylysine (Hyl) and pyridinoline cross-link (HP and LP) levels were compared between paired samples of degenerated and macroscopically normal cartilage from the same donor.



Chapter 2

AGE-RELATED ACCUMULATION OF MAILLARD REACTION PRODUCTS IN HUMAN ARTICULAR CARTILAGE COLLAGEN

Nicole Verzijl,[a,b] Jeroen DeGroot,[a,b] Esther Oldehinkel,[a] Ruud A. Bank,[a] Suzanne R. Thorpe,[c] John W. Baynes,[c] Michael T. Bayliss,[d] Johannes W.J. Bijlsma,[b] Floris P.J.G. Lafeber[b] and Johan M. TeKoppele[a]

Non-enzymatic modification of tissue proteins by reducing sugars, the so-called Maillard reaction, is a prominent feature of aging. In articular cartilage, relatively high levels of the advanced glycation endproduct (AGE) pentosidine accumulate with age. Higher pentosidine levels have been associated with a stiffer collagen network in cartilage. However, even in cartilage, pentosidine levels themselves represent < 1 cross-link per 20 collagen molecules, and as such cannot be expected to contribute substantially to the increase in collagen network stiffness. In the present study, we investigated a broad range of Maillard reaction products in cartilage collagen in order to determine whether pentosidine serves as an adequate marker for AGE levels. Not only did the well-characterized AGEs pentosidine, N^{ε}-(carboxymethyl)lysine (CML), and N^{ε}-(carboxyethyl)lysine (CEL) increase with age in cartilage collagen (all $p < 0.0001$), but also general measures of AGE cross-linking, such as browning and fluorescence (both $p < 0.0001$), increased. The levels of these AGEs are all higher in cartilage collagen than in skin collagen. As a functional measure of glycation the digestibility of articular collagen by bacterial collagenase was investigated; digestibility decreased linearly with age, proportional to the extent of glycation. Furthermore, the arginine content and the sum of the hydroxylysine and lysine content of cartilage collagen decrease significantly with age ($p < 0.0001$ and $p < 0.01$ respectively), possibly due to modification by the Maillard reaction. The observed relationship between glycation and amino acid modification has not been reported previously in vivo. *Our present results indicate that extensive accumulation of a variety of Maillard reaction products occurs in cartilage collagen with age. Altogether our results support the hypothesis that glycation contributes to stiffer and more brittle cartilage with advancing age.*

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INTRODUCTION

During aging, long-lived proteins such as collagen and eye lens proteins are nonenzymatically modified by reducing sugars. The major initial product is fructose-lysine (FL),[90,91] which results from glycation of ε-amino groups on lysine residues. In subsequent Maillard or browning reactions, products known as advanced glycation endproducts (AGEs) are formed from FL,[4,357] and accumulate with age in long-lived proteins.[25,83,90,91,295,331] These AGEs include structurally characterized adducts such as N^{ε}-(carboxymethyl)lysine (CML)[4,90,91] and N^{ε}-(carboxyethyl)lysine (CEL),[3] fluorescent cross-links, such as pentosidine formed between lysine and arginine residues,[92,295] as well as chemically unidentified compounds which result in protein-bound browning or fluorescence, and cross-linking.[185,226,290,294]

In comparison with other collagen-rich tissues such as skin, cartilage contains relatively large amounts of pentosidine.[227,295] Pentosidine levels in articular cartilage increase linearly with age,[25,83,331] as was previously described for skin collagen[93] and lens proteins.[92] Pentosidine is also present in the proteoglycans in articular cartilage,[264] but appears to be localized predominantly in the collagen component of the tissue.[25] Age-related accumulation of AGE cross-links in articular cartilage collagen may result in increased stiffening of the collagen network, as was shown after *in vitro* ribosylation of

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cartilage.[25] Thus AGE cross-linking *in vivo* may contribute to the age-related impairment of the ability of articular collagen to resist mechanically induced damage and eventually cartilage degeneration.[111,157,350]

Even though pentosidine levels in cartilage collagen are high compared with the levels found in other long-lived proteins,[227,295] the absolute number of pentosidine cross-links is less than 1 cross-link per 20 collagen molecules,[25,83] and as such cannot be expected to substantially increase collagen network stiffness. In the present study, we investigated a range of Maillard reaction products in human articular cartilage collagen to determine whether the extensive accumulation of pentosidine in articular cartilage can be confirmed by other AGEs.

FL, CML, CEL and pentosidine were measured in cartilage collagen, and their levels were compared with those previously reported for skin collagen and lens protein.[81,90,91] In addition, general measures of glycation-derived cross-links, *i.e.* browning and Maillard-type fluorescence,[226] were determined. Modification of arginine, lysine and hydroxylysine residues in cartilage collagen was also determined as a possible measure of overall glycation and AGE formation. The digestibility of articular collagen by bacterial collagenase was explored as a functional measure of cross-linking.

EXPERIMENTAL PROCEDURES

Cartilage samples

Macroscopically normal human articular cartilage was obtained *post mortem* from femoral condyles within 18 h of death. Patients had no clinical history of joint disorders. Cartilage from a total of 60 donors was used; ages ranged from 2.5 to 103 years. Different subsets of samples were used for the various assays. In a few cases (n = 6), paired cartilage and skin samples were collected. All tissue samples were stored at -20°C until analysis.

Purification of collagen

Articular cartilage collagen was purified by depleting the tissue of all proteoglycans and other non-collagenous proteins by sequential enzymatic treatment with chondroitinase ABC (Sigma), trypsin (Boehringer Mannheim), and *Streptomyces* hyaluronidase (Sigma) at 37°C, as described by Schmidt *et al.*[289] This procedure resulted in the removal of more than 97% of the total glycosaminoglycans with a minimal loss of collagen (< 1%, measured as hydroxyproline; results not shown). Skin collagen was isolated by sequential extraction for 24 h at 4°C with 1 M NaCl, chloroform/methanol (2:1, v/v), and 0.5 M acetic acid as described previously.[197]

Analytical procedures

For analysis of FL, samples of cartilage collagen (1 mg in 1 ml of 6 M HCl) were hydrolysed for 24 h at 110°C under nitrogen. CML and CEL were measured separately in 2 mg samples that were first reduced overnight at 4°C in 500 µl of 0.1 M $NaBH_4$ (Sigma) in 0.1 M sodium borate buffer (pH 9.0). To remove excess $NaBH_4$, samples were washed three times with 5 ml of deionized water and then hydrolysed in 1 ml of 6 M HCl, as described above. An aliquot (30%) of the reduced hydrolysates was removed for pentosidine and amino acid analysis, described below. The FL, CML, CEL and lysine contents of the collagen hydrolysates were measured as their *N*-trifluoroacetyl methyl esters by isotope-dilution selected ion monitoring GC-MS,[3,90] using deuterated internal standards. The FL, CML and CEL content of the collagen samples is expressed as mmol per mol of lysine residues. Historical data for lens protein and skin collagen have been determined using the same methodology as described above.[3,81,90,91]

Pentosidine content and amino acid composition were determined by HPLC as described previously.[27,28] In short, dried hydrolysates were dissolved in internal standard solution containing 10 µM pyridoxine (Sigma) and 2.4 mM homoarginine (Sigma). For pentosidine analysis, samples were diluted fivefold with 0.5% (v/v) heptafluorobutyric acid (Fluka) in 10% (v/v) acetonitrile

(Rathburn, Walkerburn, Scotland, U.K.) and analysed by HPLC.[27] Pentosidine was generously given by Professor V.M. Monnier (Case Western Reserve University, Cleveland, OH, U.S.A.), and calibrated against our pentosidine standard.[92] For amino acid analysis, an aliquot of the pentosidine samples was diluted 50-fold with 0.1 M borate buffer (pH 11.4), derivatized with 9-fluorenylmethyl chloroformate (Fluka) and analysed by HPLC.[28] The pentosidine content of the collagen samples is expressed as mmol per mol of lysine residues. The quantities of arginine and the sum of the primary amines hydroxylysine and lysine, *i.e.* (hydroxy-)lysine, are expressed as mol per mol of collagen, assuming 300 hydroxyproline residues per triple-helical collagen molecule.[27]

Collagen-linked fluorescence and browning were measured in cartilage and skin collagen (1-5 mg) digested for 2 h at 65°C with 2.5 units/ml of papain (from Papaya latex, Sigma) in 300 µl of papain buffer [50 mM phosphate buffer (pH 6.5), 2 mM L-cysteine and 2 mM EDTA].[168] Fluorescence of the papain digests was measured at λ_{ex}=360 nm and λ_{em}=460 nm in a Cytofluor II Multi-Well Plate Reader (PerSeptive Biosystems), because those filters were available for the plate reader. A high correlation was found between these fluorescence measurements and the fluorescence measured at 370 nm/440 nm (the excitation-emission maxima for AGE fluorescence;[226] r = 0.99, p < 0.0001; results not shown) in an SFM-25 fluorometer (Kontron, Milan, Italy). Browning was measured as absorption at 340 nm in a Titertek Multiskan MCC/340 plate reader (Labsystems, Helsinki, Finland). The measurements were made against papain buffer. An aliquot of the papain digests was hydrolysed in 1 ml of 6 M HCl at 110°C for 20-24 h for hydroxyproline analysis.[79,313] Both fluorescence and browning were normalized to the hydroxyproline content of the digest (papain contributed < 1% of the hydroxyproline in the digests).

Collagen digestibility was measured following the digestion of cartilage collagen for 6 h at 37°C with *Clostridium histolyticum* collagenase (CLS 2; Worthington Biochemical Corp., Freehold, NJ, U.S.A.) at a final concentration of 5 µg collagenase per mg collagen in a 50 mM Tris buffer containing 5 mM $CaCl_2$, 0.15 M NaCl, 1 µM $ZnCl_2$, 0.02% (w/v) NaN_3, and 0.01% (v/v) Brij 35. After incubation, the supernatant and remaining tissue were separated and hydrolysed in 1 ml of 6 M HCl at 110°C for 20-24 hours. The relative amount of collagen in the supernatant was estimated by measuring the amount of hydroxyproline in the hydrolysates of both the supernatant and the remaining tissue.[79,313] The mean collagenase digestibility was calculated from duplicate analyses. In a subset of the donors (n = 12), an additional cartilage collagen sample was digested with papain for measurement of browning and AGE fluorescence and pentosidine content (after hydrolysis of the papain digest) as described above, in order to correlate collagenase digestibility of articular collagen with levels of AGE cross-linking.

Statistical analysis

Linear regression analyses and paired Student's t tests were performed with SPSS version 8.0 for Windows (SPSS, Chicago, IL, U.S.A.); $p < 0.05$ was considered to represent statistically significant differences.

RESULTS

Age-related variation in levels of Maillard reaction products in articular cartilage collagen

In articular cartilage from healthy individuals, a slight, but statistically significant, age-related increase in collagen glycation (measured as FL) was found (r = 0.37, $p < 0.05$; Figure 1A). The glycation of lysine residues in cartilage collagen was about 5 mmol of FL/mol of lysine in a sample from a 2.5-year-old and increased only by an additional 23% up to the age of 80 years. Thus the extent of modification of lysine residues in cartilage collagen by FL ranged from 0.5 to 0.7% over the entire human life span.

In contrast with the marginal age-related increase in the concentration of FL, AGE concentrations per amount of lysine resi-

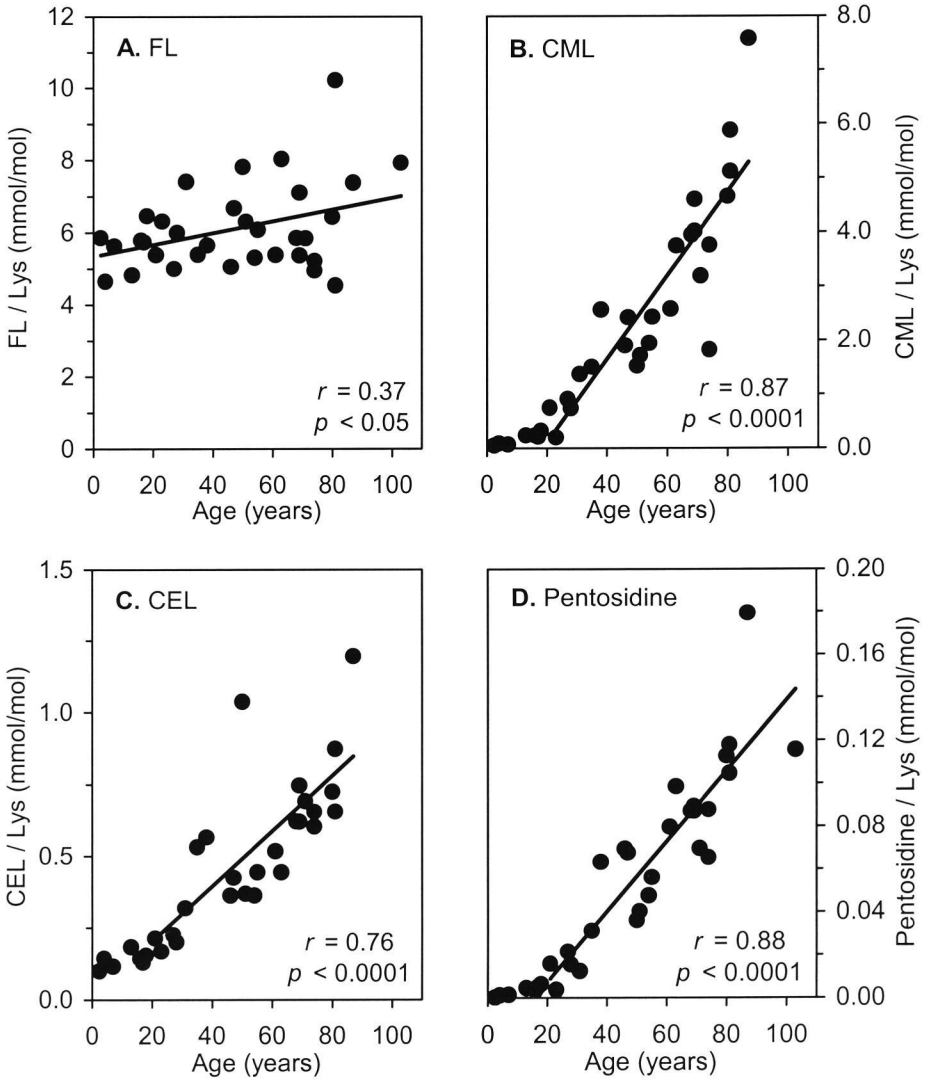


FIGURE 1 - AGEs in articular cartilage collagen increase markedly with age. (**A**) Levels of the initial glycation product FL in cartilage collagen; (**B-D**) levels of the AGEs CML, CEL and pentosidine in cartilage collagen as a function of age. Normal full-depth femoral condylar cartilage of 33 subjects in the age range 2.5-103 years was analysed; data are expressed as mmol of residues/mol of lysine. Linear regression analysis was performed on all data for FL and on data > 20 years for CML, CEL and pentosidine.

dues increased considerably with age in cartilage collagen. In immature cartilage (< 20 years), AGE levels are very low, but after maturity (> 20 years) levels of CML (r = 0.87, $p < 0.0001$; Figure 1B), CEL (r = 0.76, $p < 0.0001$; Figure 1C), and pentosidine (r = 0.88, $p < 0.0001$; Figure 1D) in cartilage collagen increased 27-fold, 6-fold and 33-fold with age respectively. When expressed per collagen triple helix, similar correlations and increases of CML, CEL and pentosidine levels with age were found (results not shown). Summed levels of CML, CEL, and pentosidine reached 5.6 mmol/mol of lysine

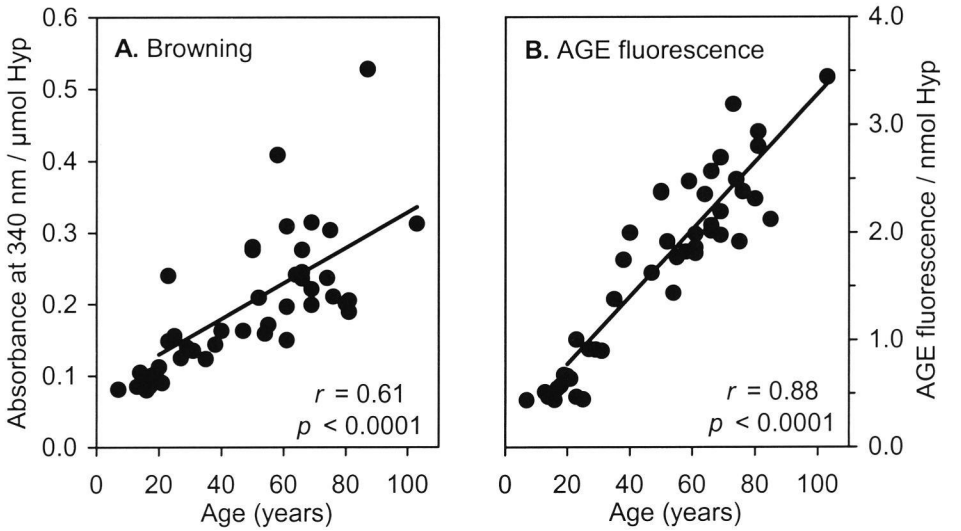


FIGURE 2 - General measures of Maillard-type cross-linking of articular cartilage collagen increase linearly with age. (**A**) Browning (absorbance at 340 nm) and AGE fluorescence (**B**) in cartilage collagen versus age. Normal full-depth femoral condylar cartilage of 45 subjects in the age range 7-103 years was analysed; data are expressed as relative units per hydroxyproline. Linear regression analysis was performed on data > 20 years.

in 80-year-old cartilage collagen (approx. 0.6% of the total lysine residues in cartilage collagen).

Besides the concentrations of the chemically characterized AGEs, the levels of general measures of Maillard-type cross-linking were also determined. Both browning (absorbance at 340 nm) and AGE fluorescence increased significantly with age in cartilage collagen ($r = 0.61$, $p < 0.0001$; Figure 2A and $r = 0.88$, $p < 0.0001$; Figure 2B respectively). The threefold increase in browning and the fivefold increase in fluorescence from age 20 to 80 were less pronounced than the age-related increases in CML, CEL, and pentosidine (Figures 1B-1D).

With increasing age, a decrease in the arginine, hydroxylysine and lysine content of cartilage collagen was observed. While the decrease in lysine content was not significant, the arginine content and the sum of the primary amines decreased significantly with age ($r = -0.66$, $p < 0.0001$ and $r = -0.47$, $p < 0.01$ respectively; Figure 3). Over the entire human life span (0-80 years), 15 arginine residues (9.5%) and seven (hydroxy-)lysine residues (6.9%) are lost per cartilage collagen molecule, suggesting that they have been subjected to modification during aging. Both the sum of three well-characterized AGEs (CML, CEL and pentosidine) and the AGE fluorescence correlated significantly with the mean degree of modification of arginine, hydroxylysine and lysine residues in collagen ($r = 0.58$, $p < 0.001$ and $r = 0.52$, $p < 0.05$ respectively).

Besides the age-related increase in Maillard reaction products, we also explored functional properties of articular collagen (*i.e.* enzymatic digestibility) as a measure of glycation cross-links. An age-related decline in digestibility of cartilage collagen by bacterial collagenase of 0.3% per year was observed ($r = -0.75$, $p < 0.0001$; Figure 4A), which may well be indicative of increased AGE cross-linking with advancing age. Correspondingly, the collagenase digestibility showed a strong negative correlation with the pentosidine content ($r = -0.87$, $p < 0.0005$), browning ($r = -0.82$, $p < 0.005$), and AGE fluorescence ($r = -0.87$, $p < 0.0005$; Figure 4B) of the collagen.

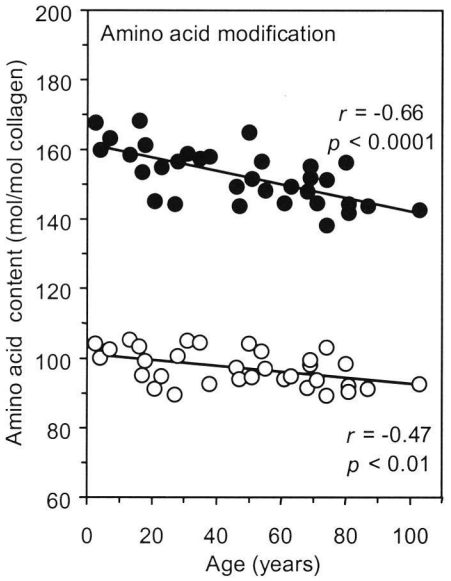
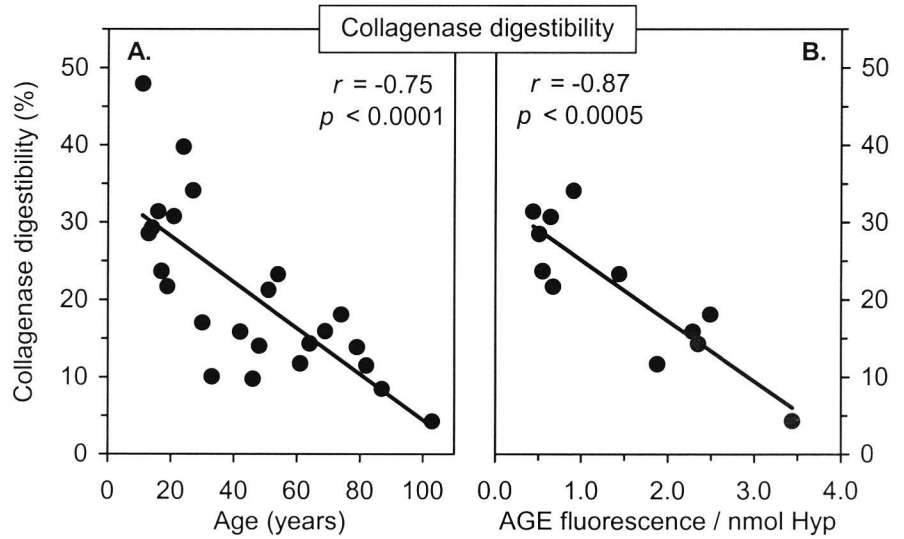


FIGURE 3 - Arginine and (hydroxy-)lysine content of articular cartilage collagen decrease with age. Arginine (●) and (hydroxy-)lysine (○; sum of hydroxylysine and lysine) content of cartilage collagen as a function of age. Normal full-depth femoral condylar cartilage of 33 subjects in the age range 2.5-103 years was analysed; data are expressed as mol of residues/mol of collagen, assuming 300 hydroxyproline residues per triple helix. Linear regression analysis was performed on all data.

Comparison of Maillard reaction products in cartilage collagen with those in skin collagen and lens protein

Levels of the chemically well-characterized AGEs in cartilage collagen from elderly donors were compared with previously reported data on human skin collagen and lens protein.[3,81,90,91] The pentosidine level in aged cartilage collagen was fourfold higher than in skin collagen and 25-fold higher than in lens protein from donors of a comparable age (Table 1). This comparison confirms previously published data that pentosidine levels in cartilage are extremely high.[227,295] Furthermore, CML and CEL concentrations in cartilage collagen are higher than in skin collagen (2.5-fold and 1.4-fold respectively; Table 1). As a general measure of AGE cross-linking, Maillard-type fluorescence was measured in paired samples of cartilage and skin collagen of aged donors (n = 6, age 76.4 ± 4.4 years; means ± S.D.). The fluorescence in cartilage collagen (36.6 ± 3.7 relative fluo-

FIGURE 4 - Digestibility of articular cartilage collagen by bacterial collagenase decreases with age and with AGE cross-linking. The proportion of cartilage collagen digested by *C. histolyticum* collagenase **(A)** as a function of age (n = 24, age range 11-103 years) and **(B)** as a function of AGE cross-linking determined as Maillard-type fluorescence (see the Experimental section; n = 12, age range 13-103 years). Linear regression analysis was performed on all data.



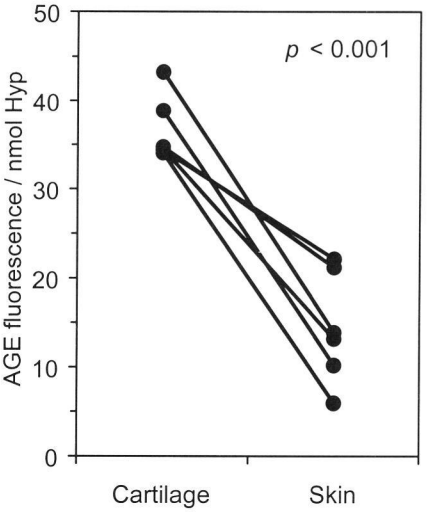


FIGURE 5 - Maillard-type fluorescence is higher in cartilage compared with skin collagen. AGE fluorescence was measured in paired samples of cartilage and skin collagen of aged donors (n = 6, age 76.4 ± 4.4 years; mean ± S.D.). Data are expressed as relative units per hydroxyproline. The significance of the difference between cartilage and skin collagen was tested using a paired Student's *t* test.

rescence units/nmol of hydroxyproline) was about 2.5-fold higher than in skin collagen (14.4 ± 6.3 relative fluorescence units/nmol of hydroxyproline, $p < 0.001$; Figure 5). Thus AGE levels in general are severalfold higher in cartilage collagen than in skin collagen. Although pentosidine levels in cartilage collagen are as much as 25-fold higher than in the lens, CML and CEL levels in aged cartilage collagen are comparable with (CML) or even lower (CEL) than in lens protein from elderly donors.

In addition to their absolute levels in tissues from elderly donors, the mean CML/CEL ratio was compared between cartilage collagen, skin collagen and lens protein, because this ratio may yield insight into the chemical origin of these lysine modifications in proteins. The highest CML/CEL ratio (6.06) was measured in cartilage collagen, being 1.8-fold higher than in skin collagen (3.40) and 4.6-fold higher than in lens protein (1.31; Table 1).

DISCUSSION

Maillard reaction products in articular cartilage collagen

There have been only limited studies of non-enzymatic glycation and AGE accumulation in human articular cartilage.[25,83,264,319,331] Most of the relevant studies have focused on pentosidine, a well-known marker for advanced glycation reactions,[92,295] which is present in cartilage collagen in relatively high amounts compared with other tissues.[227,295] Recently, the AGEs CML and CEL were shown to be present in human skin collagen

TABLE 1 - Comparison of cartilage collagen levels of Maillard reaction products in elderly subjects with previously reported data for skin and lens. Levels of Maillard reaction products expressed as mmol/mol of lysine (means ± S.D., if available). Cartilage collagen data are from the present study and represent an age range of 61-87 years (n = 12). FL data for skin collagen and lens protein (mean for ages 60-85 years) are taken from Ref. 90 and 91. The levels of AGEs in skin collagen (85-year-old donor pool) and lens protein (ages 60-85 years) are reproduced from Ref. 81.

	Content (mmol/mol)		
	Cartilage collagen	Skin collagen	Lens protein
FL	6.36 ± 1.60	5.12	1.57
Pentosidine	0.098 ± 0.030	0.025 ± 0.004	0.004 ± 0.002
CML	4.24 ± 1.51	1.70 ± 0.31	4.95 ± 0.50
CEL	0.70 ± 0.19	0.50 ± 0.09	3.78 ± 0.28
CML/CEL	6.06	3.40	1.31

and lens protein at concentrations considerably higher than that of pentosidine.[3,90,91] In the present study, we also demonstrate the presence and age-related accumulation of CML and CEL in cartilage collagen in a broader study of Maillard reaction products in articular cartilage collagen.

Glycation (measured as FL) of articular cartilage collagen is relatively constant throughout adult life. During the first two decades of life, low levels of the AGEs CML, CEL and pentosidine are measured. This suggests that cartilage collagen undergoes relatively rapid turnover during this phase of life, so that AGEs are efficiently removed and do not accumulate.[25] After maturity has been reached, the concentrations of the AGEs increase up to 30-fold with age, consistent with the slow turnover reported for collagen in adult cartilage.[25,208] As expected, CML and CEL are present in cartilage collagen at considerably higher concentrations than pentosidine, as was previously described for skin collagen and lens protein.[3,90,91] Not only the well-characterized AGEs, but also general measures of AGE content, browning and Maillard-type fluorescence, increased with donor age.

Even though the levels of CML, CEL and pentosidine are relatively high in cartilage collagen compared with those in skin collagen,[90,93] and the level of pentosidine is high compared with that in lens protein,[3,91] it is not possible to draw conclusions on the extent of cartilage collagen modification solely based on the levels of these AGEs, because these analyses are limited to the few AGEs that are chemically characterized. Therefore we measured the cartilage collagen content of amino acids that become modified during glycation and AGE formation. Both the arginine and (hydroxy-)lysine content of cartilage collagen decrease significantly with age, implying an age-related increase in amino acid modification. The age-related modification of amino acids can be due to several processes, including glycation and lipid oxidation.[115,223] Furthermore, the modification of (hydroxy-)lysine residues can also be the result of enzymatic collagen cross-linking. Mature pyridinoline cross-links comprise over 90% of the presently characterized enzymatic cross-links in type II collagen in articular cartilage.[96] Inasmuch as the extent of cross-linking of cartilage collagen by mature pyridinoline cross-links remains constant throughout the entire lifespan,[25] it is expected that the age-related increase in modification of both arginine and (hydroxy-)lysine amino acids in cartilage collagen results from glycation or oxidation processes. Based on our present results, only 4.7% of the age-related loss of (hydroxy-)lysine residues can be explained by the well-characterized AGEs CML, CEL and pentosidine. Still, this is consistent with data from *in vitro* incubations of cartilage collagen with ribose or threose in which respectively 3.9% and 1.1% of the modified (hydroxy-)lysine residues were identified as CML, CEL, or pentosidine (N. Verzijl, J. DeGroot, R. A. Bank, A. Maroudas, S. R. Thorpe, J. W. Baynes, J. W. J. Bijlsma, F. P. J. G. Lafeber and J. M. TeKoppele, unpublished work). In acid hydrolysates of skin collagen[221] and lens protein,[73,86,91] no age-related changes in arginine and (hydroxy-)lysine content of the protein are detected, implying that, unlike cartilage collagen, the age-related accumulation of acid-stable modifications is not high in these proteins. Thus not only on the basis of the high AGE levels in cartilage collagen, but also on the basis of amino acid measurements, it is suggested that cartilage collagen is extensively modified by glycation.

The fact that the solubility of human skin collagen by enzymatic digestion with pepsin decreases with age is attributed to the increased AGE cross-linking with advancing age.[290,291] Similarly, our data show that the digestibility of articular cartilage collagen by bacterial collagenase decreases substantially with age: from approx. 30% in 20-year-old cartilage to 10% in 80-year-old cartilage. Inasmuch as the collagenase digestibility of cartilage collagen is strongly correlated with its pentosidine content and AGE fluorescence (Figure 4B), the age-related decrease in digestibility is most likely due to the increase in AGE cross-linking with age.

Maillard reaction products in cartilage compared with skin and lens

It is assumed that the extent of glycation of long-lived tissue proteins is in equilibrium with the tissue glucose concentration.[90] This assumption is based on the 3-4-fold higher extent of glycation of skin collagen[90] compared with lens protein,[91] which is consistent with the higher glucose concentration in plasma and skin extravascular fluid (approx. 5 mM) than in the lens (1-2 mM).[90,134,184,327] In articular cartilage, the glucose concentration is equal to the plasma glucose concentration.[201] Therefore it was not surprising that the level of glycated lysine residues in cartilage collagen (5-7 mmol of FL/mol of lysine) is comparable with that in skin collagen[90] (Table 1).

Throughout life, skin collagen contains lower levels of CML and CEL compared with FL.[90] In lens, the CML and CEL content is considerably higher than its FL content.[3,91] As proposed by Dunn *et al.*,[90] this indicates that skin collagen undergoes relatively more initial glycation than lens protein, but is exposed to less oxidative stress. The last finding is corroborated by the much lower levels in skin collagen compared with lens protein of *o*-tyrosine, a marker of oxidative damage to tissue proteins.[355,356] In cartilage collagen, as in skin collagen, lower levels of CML and CEL are present than of FL, suggesting that in cartilage oxidative processes may also play a less important role than in the lens. This is consistent with the low oxygen tension of articular cartilage, due to the avascular nature of the tissue.[24,203] In general, the results suggest that parameters determining concentrations of CML and CEL relative to the initial glycation product FL, such as tissue glucose concentration and oxidative stress, are relatively similar for cartilage and skin collagen, and different from lens protein. However, AGE levels are also dependent on protein turnover rates.[59,227,295,332] Lens protein[91,130] and cartilage collagen[208] essentially do not turn over, whereas skin collagen has a turnover faster than cartilage and lens.[291] While cartilage and skin collagen are relatively comparable in glucose concentrations and oxidative stress as determinants of AGE levels, the difference in protein turnover is clearly reflected in the higher CML, CEL and pentosidine levels in cartilage compared with skin collagen (Table 1).

Ahmed *et al.*[3] indicated that the CML/CEL ratio in different tissues provides insight into the chemical origin of these lysine modifications in proteins. In cartilage collagen a higher CML/CEL ratio (approx. 6.1) was observed compared with skin collagen (approx. 3.4) and lens protein (approx. 1.3). The approximate 1:1 ratio in lens proteins was close to the CML/CEL ratio obtained with 3-deoxyglucosone and ascorbate upon incubation of collagen with these carbohydrates *in vitro*.[3] In cartilage collagen the CML/CEL ratio is closer to the ratios found after *in vitro* incubation with ribose, glucosone and also glucose. A high CML/CEL ratio in cartilage and skin collagen compared with lens protein is consistent with the higher glucose concentration in plasma compared with the lens,[90] and with the higher ascorbate than glucose concentration in the lens.[134,163,321,327] In addition, the ability of ascorbate to glycate and cross-link lens proteins is much greater than that of glucose.[184] These observations suggest that glycation and AGE formation in cartilage and skin collagen is more dependent on glucose, whereas ascorbate may be more important in these processes in the lens.

From our present results it is clear that, in human cartilage collagen, not only pentosidine levels, but also the levels of other well-characterized AGEs and general measures of AGE cross-linking, substantially increase with age. Predominantly on the basis of the 5-10% decrease in arginine and (hydroxy-)lysine content of cartilage collagen during the human lifespan, we conclude that overall cartilage collagen modification by glycation is relatively high compared to other long-lived human proteins, and that pentosidine serves as an adequate marker for overall AGE levels. The age-related accumulation of high levels of AGEs in articular cartilage collagen may contribute to the

observed stiffening of the cartilage collagen network with advancing age.[25,122] The increased stiffness might make the cartilage collagen network more brittle, as described for vascular tissues,[304] and as such impair the resistance of articular collagen to fatigue and predispose aged cartilage to damage and eventual degeneration. Thus the accumulation of AGEs represents a conceivable molecular mechanism whereby age is a predisposing factor for the development of osteoarthritis.[25]

ACKNOWLEDGEMENTS

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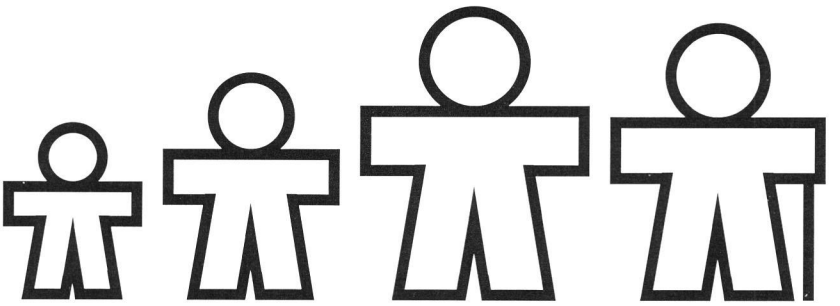

Chapter 3

Effect of Collagen Turnover on the Accumulation of Advanced Glycation Endproducts

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Collagen molecules in articular cartilage have an exceptionally long life-time, which makes them susceptible to the accumulation of advanced glycation endproducts (AGEs). In fact, in comparison to other collagen-rich tissues, articular cartilage contains relatively high amounts of the AGE pentosidine. To test the hypothesis that this higher AGE accumulation is primarily the result of the slow turnover of cartilage collagen, AGE levels in cartilage and skin collagen were compared with the degree of racemization of aspartic acid (%D-Asp, a measure of the residence time of a protein). AGE (N^{ε}-(carboxymethyl)lysine, N^{ε}-(carboxyethyl)lysine, and pentosidine) and %D-Asp concentrations increased linearly with age in both cartilage and skin collagen ($p < 0.0001$). The rate of increase in AGEs was greater in cartilage collagen than in skin collagen ($p < 0.0001$). %D-Asp was also higher in cartilage collagen than in skin collagen ($p < 0.0001$), indicating that cartilage collagen has a longer residence time in the tissue, and thus a slower turnover, than skin collagen. In both types of collagen, AGE concentrations increased linearly with %D-Asp ($p < 0.0005$). Interestingly, the slopes of the curves of AGEs versus *%D-Asp,* i.e. *the rates of accumulation of AGEs corrected for turnover, were identical for cartilage and skin collagen. The present study thus provides the first experimental evidence that protein turnover is a major determinant in AGE accumulation in different collagen types. From the age-related increases in %D-Asp the half-life of cartilage collagen was calculated to be 117 years and that of skin collagen 15 years, thereby providing the first reasonable estimates of the half-lives of these collagens.*

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Introduction

Nonenzymatic glycation is a post-translational modification of proteins *in vivo*, which is initiated by the spontaneous reaction of sugars with lysine residues in proteins and eventually results in the formation of advanced glycation endproducts (AGEs), such as N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) and pentosidine.[3,91,295] Because AGEs are irreversible chemical modifications of protein, they accumulate with age in long lived proteins such as lens crystallins and tissue collagens.[25,83,90,91,196,295,331,340] Because collagen molecules in articular cartilage have an exceptionally long lifetime (>100 years),[203,208] they are highly susceptible to the accumulation of AGEs. Indeed, in comparison to other collagen-rich tissues (such as skin), articular cartilage contains relatively high amounts of pentosidine.[227,295] Although differences in AGE levels between different proteins have been attributed to differences in protein turnover rates,[59,227,295,332] no quantitative evidence to support this assumption is available.

To compare protein turnover rates, information on the residence time of a protein in tissue can be obtained from the racemization of aspartic acid. Amino acids are incorporated into peptides and proteins as the L-enantiomers. During aging, racemization slowly converts the L-form into a racemic mixture of D- and L-forms. Aspartic acid is one of the fastest racemizing amino acids,[135,210] so that D-aspartic acid can be detected in proteins that turn over slowly. There is a close relationship between

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chronological age and D-aspartic acid accumulation in the white matter of the brain,[199] the eye lens,[210] dentin,[136] and bone.[247,260] Furthermore, several studies have shown the age-related accumulation of D-aspartic acid in cartilagenous tissues such as the intervertebral disc,[280] rib cartilage,[261] and articular cartilage.[204,208] Measurement of aspartic acid racemization has proven to be a valuable tool to derive quantitative information on protein residence time, *i.e.* protein turnover.[204,208]

The present study was designed to establish the effect of protein turnover rate on the kinetics of accumulation of CML, CEL, and pentosidine in cartilage and skin collagen. Differences in the rate of accumulation of these AGEs were then corrected for differences in residence times of these collagens in the tissue, estimated from the degree of racemization of aspartic acid. The results indicate that differences in levels of AGEs in cartilage and skin collagens may be almost solely attributed to differences in the rate of turnover of these proteins.

EXPERIMENTAL PROCEDURES

Tissue samples

Macroscopically normal human articular cartilage was obtained *post mortem* from femoral condyles within 18 h after death from patients who had no clinical history of joint disorders or diabetes (n = 23). Skin samples were obtained from the medial aspect of the buttock of nondiabetic donors (n = 27; healthy volunteers or at autopsy within 6 h after death). In a few additional cases, paired cartilage and (knee) skin samples (n = 6; age 76.4 ± 4.4 years; mean ± S.D.) were obtained at autopsy within 18 h after death. Bovine articular cartilage and skin were obtained from the metacarpophalangeal joint of a 6-month-old calf. All samples were stored at -20°C until analyzed.

Purification of collagen

Articular cartilage collagen was isolated by depleting the tissue of all proteoglycans and other non-collagenous proteins using sequential enzymatic treatment with chondroitinase ABC (Sigma), trypsin (Roche Molecular Biochemicals), and *Streptomyces* hyaluronidase (Sigma) for 20 h at 37°C as described by Schmidt *et al.*[289] This procedure resulted in the removal of more than 97% of the glycosaminoglycans with a minimal loss of collagen (<1%, measured as hydroxyproline). Skin collagen was isolated by sequential extraction for 24 h at 4°C with 1 M NaCl, chloroform/methanol (2:1), and 0.5 M acetic acid as described previously.[90] All collagen samples were lyophilized following purification.

CML, CEL, and pentosidine analyses

For analysis of CML and CEL, 2 mg of dry collagen was reduced overnight at 4°C in 500 µl of 0.1 M $NaBH_4$ (Sigma) in 0.1 M sodium borate buffer (pH 9.0). To remove excess $NaBH_4$, the samples were washed 3 times with 5 ml of deionized water and then hydrolyzed in 1 ml of 6 M HCl for 24 h at 110°C under nitrogen. After hydrolysis, an aliquot of these samples (30%) was removed for pentosidine analysis. CML, CEL, and lysine content of the collagen hydrolysates were simultaneously measured as their *N*-trifluoroacetylmethyl esters by isotope dilution-selected ion monitoring gas chromatography-mass spectrometry,[3,90] using deuterated internal standards. Pentosidine was determined in collagen hydrolyzates by reversed-phase high-performance liquid chromatography, as described previously.[27,92] The CML, CEL, and pentosidine content of the collagen samples are expressed as µmol or mmol per mol of lysine residues.

Percentage D-Asp in collagen

The percentage D-Asp (%D-Asp) in cartilage and skin collagen was determined by high-performance liquid chromatography according to Aswad[17] with slight modifications. In short, collagen samples (1-2 mg dry weight) were digested for 2 h at 65°C with 5 units/ml papain (from Papaya latex, Sigma) in 300 µl of papain buffer (50 mM phosphate buffer (pH 6.5), 2 mM L-cysteine, and 2 mM EDTA).[168] An aliquot of the papain digests (50 µl) was subsequently hydrolyzed in 1 ml

of 6 M HCl at 100°C for 4 h. After drying, the hydrolysates were dissolved in 1 ml of 0.1 M sodium borate buffer (pH 9.5) of which 20 µl was derivatized with *o*-pthaldialdehyde/*N*-acetyl-L-cysteine (Sigma) in a MIDAS autosampler (Spark Holland, Emmen, The Netherlands). Derivatized D- and L-Asp were separated on a C18 column (TSKgel ODS-80TM, 150 mm x 4.6 mm, 5-µm particle size; TosoHaas, Stuttgart, Germany) at a flow rate of 0.8 ml/min. Solvent A was 50 mM sodium acetate (pH 5.9). Solvent B was 80% (v/v) methanol (Rathburn, Walkerburn, U.K.) and 20% (v/v) solvent A. Elution of D- and L-Asp was achieved in two steps as follows: isocratic elution with 9% (v/v) solvent B for 5 min and then a linear increase in the content of solvent B to 100% over a period of 5 min. The column was washed for 10 min with 100% solvent B and equilibrated for 10 min in 9% (v/v) solvent B before injecting the next sample. Fluorescence was monitored at 340/440 nm. Standard solutions of either D- or L-Asp were calibrated using nonchiral FMOC (9-fluorenylmethyl chloroformate) derivatization as described previously[28] and mixed into standards of known %D-Asp.

All of %D-Asp data were corrected for the amount of D- and L-Asp present in papain (5-10% of the aspartic acid in the samples) and for racemization during the hydrolysis step. The latter was performed either by subtracting the intercept of the graph for %D-Asp *versus* donor age (for the cartilage and skin collagen age ranges) or by subtracting the %D-Asp measured in young bovine cartilage and skin collagen (for the paired cartilage and skin samples of n = 6 aged donors), with both methods resulting in comparable corrections.

Measurement of *in vitro* rate of aspartic acid racemization in different collagen types

In order to estimate the rate of aspartic acid racemization in cartilage collagen (type II) and in skin collagen (type I), a heating experiment on young bovine cartilage and skin collagen was performed as described by Ohtani.[245] Dry collagen samples (2-7 mg) were placed in glass test tubes and heated at 120, 140, or 160°C for 15-120 min. Samples were digested in papain (5 units/ml in papain buffer, 100 µl per mg of collagen), and the racemization of aspartic acid (*i.e.* the D/L-Asp ratio) was determined after hydrolysis, as described above.

Amino acid racemization follows first-order kinetics described by Equation 1,[18,307]

$$\ln((1+D/L)/(1-D/L))_t = 2k_{Asp}.t + \ln((1+D/L)/(1-D/L))_{t=0} \quad \text{(Eq. 1)}$$

in which D/L is the ratio of D- to L-Asp; t is any given time during racemization; k_{Asp} is the rate constant of aspartic acid racemization, and the logarithmic term at t = 0 describes the amount of D-Asp measured in unheated collagen. The Arrhenius equation was used to determine the activation energy for the racemization of aspartic acid residues in each type of collagen. This equation describes the quantitative relation between temperature and reaction rate[14] as shown in Equation 2,

$$\ln[k_{Asp}] = -Ea/(RT) + \text{constant} \quad \text{(Eq. 2)}$$

in which Ea is the activation energy; R is the gas constant; and T is the absolute temperature. The racemization rate constant (k_{Asp}(h)) was determined at each temperature by using Equation 1 and by using only the data that showed a linear increase in ln((1+D/L)/(1-D/L)) with time (defined as a correlation constant $r > 0.95$ in linear regression analysis). The ln[k_{Asp}(h)] values were plotted against the corresponding reciprocals of the absolute temperature to obtain a linear transformation of the Arrhenius equation.

Statistical analysis

Linear regression analysis, analysis of covariance (ANCOVA), and paired Student's *t* tests were performed with SPSS version 8.0 for Windows (SPSS, Chicago); $p < 0.05$ was considered to represent statistically significant differences.

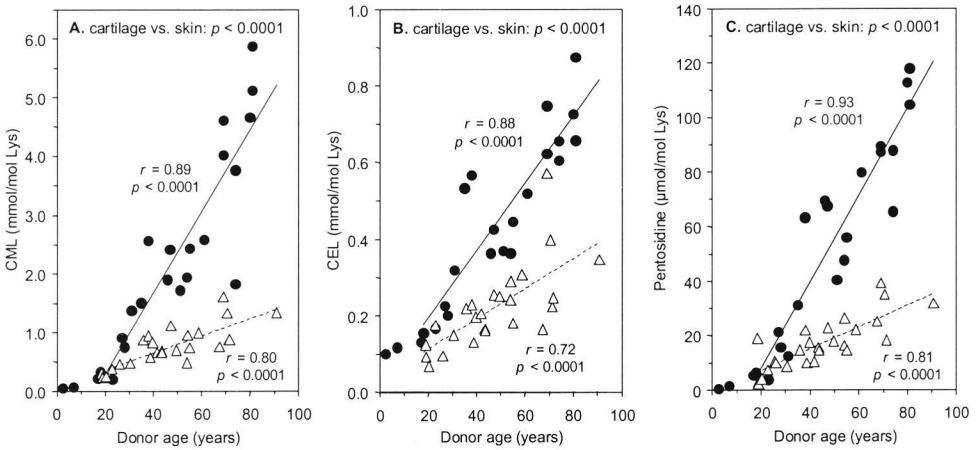


FIGURE 1 - AGEs accumulate linearly with chronological age in both cartilage and skin collagen, but the accumulation rate is higher in cartilage collagen. A-C show the age-dependent increase in the concentration of CML (**A**), CEL (**B**), and pentosidine (**C**) in human articular cartilage collagen (●) and skin collagen (△). Normal full depth femoral condylar cartilage of 20 subjects in the age range 3-81 years and normal skin from the medial aspect of the buttock of 26 subjects in the age range 19-91 years were analyzed. Linear regression analysis and ANCOVA were performed on cartilage (solid line) and skin (dashed line) collagen data ≥18 years.

RESULTS AND DISCUSSION

In the present study, AGE levels in cartilage and skin collagen were compared with the degree of racemization of aspartic acid, a measure of the residence time of a protein,[72,118,135,204,208] to provide quantitative data on the relationship between turnover rates and AGE accumulation.

AGE levels in human cartilage collagen and skin collagen

After maturity has been reached (> 18 years), CML levels increase linearly with age in both human articular cartilage collagen ($r = 0.89$, $p < 0.0001$; Figure 1A) and skin collagen ($r = 0.80$, $p < 0.0001$; Figure 1A). The rate of CML accumulation with age is significantly higher in cartilage collagen than in skin collagen (ANCOVA with age as covariant; $p < 0.0001$). Consistent with the results for CML, the levels of CEL and pentosidine in cartilage and skin collagen also increase linearly with age ($p < 0.0001$ in all cases; Figure 1, B and C), with the rate of accumulation being higher in cartilage than in skin collagen ($p < 0.0001$ for both; Figure 1, B and C). Taken together, our data on three different AGEs consistently show that AGE levels in cartilage collagen increase more rapidly with age and eventually reach higher levels than in skin collagen. In the late 1980s, Sell and Monnier[295] already reported higher levels of pentosidine in cartilage compared to skin from elderly subjects and suggested that this difference may be due to a higher turnover of skin. Up to this time, no quantitative data on protein turnover have been related to AGE levels to substantiate this suggestion.

Rate constant of racemization of aspartic acid in cartilage and skin collagen

Using %D-Asp levels in cartilage and skin collagen to compare the residence time of collagen in these two tissues assumes that the intrinsic rate constant of aspartic acid racemization at body temperature is identical in both collagens. Because differences in the rate of aspartic acid racemization have been shown to exist between collagens from other sources, e.g. bone and dentin,[245,246] a comparison of these rates in cartilage and skin collagen was necessary.

Therefore, the relation between the rate constant of racemization of L-Asp into D-Asp and temperature was determined for both cartilage and skin collagen in an *in vitro* heating experiment. The Arrhenius equations, describing the quantitative relation between the rate constant of racemization of aspartic acid (k_{Asp}) and the reciprocal of the absolute heating temperature (1/T), are shown in Figure 2. This relation is identical for cartilage and skin collagen (ANCOVA with 1/T as covariant; $p = 0.58$). The temperatures of human knee cartilage and skin are lower than 37°C but comparable; the intra-articular temperature in the knee joint is 31.7-33.9°C,[126,147] and the mean body skin temperature is 32.6°C.[69] Extrapolating the Arrhenius equations to 33°C shows that the respective rate constants for aspartic acid racemization in cartilage and skin collagen in the human body are 1.78×10^{-6} and 1.71×10^{-6} per year, respectively. Consequently, the %D-Asp levels in cartilage and skin collagen can be used validly as a measure of residence time of the collagen in the tissue.

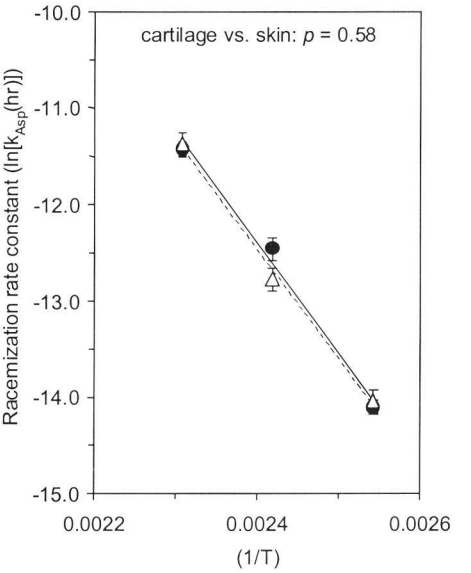


FIGURE 2 - The intrinsic rate constant for racemization of aspartic acid is identical in cartilage and skin collagen. Arrhenius plot showing the identical relation between the rate constant for racemization of aspartic acid ($k_{Asp}(h)$) and the reciprocal of the absolute temperature (1/T) for bovine cartilage (●) and skin (△) collagen, determined in a heating experiment as described under "Experimental Procedures". Data are mean ± S.D. The Arrhenius equation for cartilage (solid line) and skin (dashed line) were $\ln(k_{Asp}(h)) = -11{,}347\ (1/T) + 14.77$ and $\ln(k_{Asp}(h)) = -11{,}472\ (1/T) + 15.13$), respectively.

Racemization of aspartic acid in human cartilage collagen and skin collagen

Inasmuch as the intrinsic rate constant of aspartic acid racemization at body temperature is identical in human articular cartilage collagen and skin collagen, rates of turnover of these collagens were compared by measuring the degree of racemization of aspartic acid. In both cartilage collagen and in skin collagen, the racemization of aspartic acid (%D-Asp) increases linearly with age ($r = 0.95$, $p < 0.0001$, and $r = 0.78$, $p < 0.0001$, respectively; Figure 3). Like the rate of AGE accumulation (Figure 1), the rate of D-Asp accumulation is also higher in cartilage collagen than in skin collagen (ANCOVA with age as covariant; $p < 0.0001$). The latter implies that cartilage collagen has a longer residence time in the tissue, *i.e.* a slower turnover, than skin collagen that may contribute to the observed higher AGE levels in cartilage collagen.

Relation between collagen AGE levels and residence time

The goal of the present study was to establish the effect of protein turnover rate on the accumulation of AGEs in cartilage and skin collagen. The rate of AGE accumulation was higher in cartilage collagen than in skin collagen (Figure 1, A-C) and cartilage collagen had a longer residence time in the tissue, and thus a slower turnover, than skin collagen (Figure 3). These results strongly suggest that turnover is an important determinant of the rate of AGE accumulation in proteins. Incubation of collagen in high glucose under aerobic conditions[356] did not induce aspartic acid racemization (N. Verzijl, J. DeGroot, J.M. TeKoppele, S.R. Thorpe, and J.W. Baynes, manuscript in preparation),

TABLE I - D-Asp accumulation in human collagen. Comparison of the *in vivo* rate constants of D-aspartic acid accumulation with age in the collagen fraction of different human collagen-containing tissues.

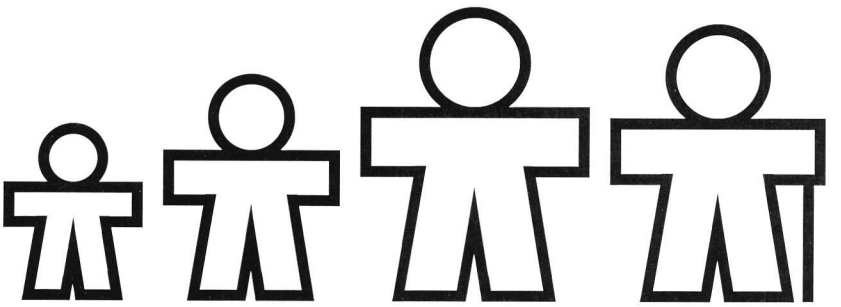
Tissue	k_{Asp} (x 10^{-4}/year)	Half-life (years)
Skin	1.17	14.8
Articular cartilage	2.63	117
Articular cartilage[208]	2.74	174
Dentin[248]	5.00	> 500

rate of D-Asp accumulation in cartilage collagen as determined in the present study is close to the rate in cartilage collagen as determined by Maroudas *et al.*[208] Because Table I compares collagens with similar intrinsic aspartic acid racemization rates, these *in vivo* rate constants of D-Asp accumulation probably reflect the rate of turnover of the protein. Therefore, we conclude that the rate of collagen turnover is higher in skin than in cartilage (see above), whereas dentin collagen has the slowest rate of turnover.

In summary, we have shown that in both cartilage and skin collagen AGE levels (CEL, CML, and pentosidine) are linearly related to levels of aspartic acid racemization, a direct measure of protein residence time, implying that protein turnover is an important factor in the accumulation of AGEs. Furthermore, consistent with a slower turnover of cartilage collagen compared with skin collagen, both %D-Asp levels and AGE levels are higher in cartilage collagen than in skin collagen. As a result, the rate of accumulation of AGEs corrected for turnover (based on %D-Asp) is similar for cartilage and skin collagen, which provides direct experimental evidence that protein turnover is a major determinant of AGE accumulation.

ACKNOWLEDGEMENTS

This study was supported by grants from the Netherlands Organization for Scientific Research (NWO), the Dutch Arthritis Association, NIDDKD Grant DK 19971 from the National Institutes of Health, National Institutes of Health Grant EY 10697, and the Diabetes Research and Wellness Foundation (Fairfax, VA). We thank Mark Sochaski (University of South Carolina, Columbia, SC), Tom VanDenBroek and Esther Oldehinkel (TNO Prevention and Health, Leiden, The Netherlands) for excellent technical assistance.



Chapter 4

AGE-RELATED ACCUMULATION OF THE ADVANCED GLYCATION ENDPRODUCT PENTOSIDINE IN HUMAN ARTICULAR CARTILAGE AGGRECAN; THE USE OF PENTOSIDINE LEVELS AS A QUANTITATIVE MEASURE OF PROTEIN TURNOVER

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During aging, nonenzymatic glycation results in the formation and accumulation of the advanced glycation endproduct pentosidine in long-lived proteins, such as articular cartilage collagen. In the present study, it was investigated whether pentosidine accumulation also occurs in cartilage aggrecan despite its higher turnover than collagen. Furthermore, pentosidine levels in aggrecan subfractions of different residence time were used to explore pentosidine levels as a quantitative measure of aggrecan turnover. In order to compare protein turnover rates, protein residence time was measured as racemization of aspartic acid. Pentosidine levels increase with age in cartilage aggrecan, as has previously been shown for collagen. Consistent with the faster turnover of aggrecan (compared to collagen), the rate of pentosidine accumulation was 3-fold lower in aggrecan than in collagen. In the subfractions of aggrecan, pentosidine levels increased with protein residence time. These pentosidine levels were used to estimate the half-life of the globular hyaluronan binding domain of aggrecan to be 19.5 years, which is in good agreement with the estimated half-life of 23.5 years based on aspartic acid racemization. In aggrecan from osteoarthritic (OA) cartilage decreased pentosidine levels were found compared to normal cartilage, which reflects increased aggrecan turnover during the OA disease process. In conclusion, we showed that pentosidine accumulates with age in aggrecan and that pentosidine levels can adequately be used as a measure of turnover of long-lived proteins, both during normal aging and during disease.

SUBMITTED FOR PUBLICATION

INTRODUCTION

Nonenzymatic modification of tissue proteins by reducing sugars is a prominent feature of aging. These sugar-derived modifications, known as advanced glycation endproducts (AGEs), accumulate with age in long-lived proteins.[25,83,90,295] Once formed, they are only removed when the protein is removed. Pentosidine, a cross-link resulting from lysine, a sugar, and arginine, is one of the best characterized AGEs and is considered an adequate marker for the process of nonenzymatic glycation.[92,295,340] Pentosidine levels in collagen differ considerably between tissues: e.g. cartilage collagen contains three-fold higher pentosidine levels than skin collagen.[227,295,341] Recently, we have been able to quantitatively relate AGE accumulation to rates of collagen turnover in these two tissues.[341]

The two major extracellular matrix proteins in articular cartilage, collagen and aggrecan, differ largely in structure and function,[176,233] and - most importantly - turnover rate.[208] The half-life of collagen in healthy human cartilage is estimated to be more than 100 years,[208,341] meaning that in normal adult cartilage collagen turnover is virtually absent. In contrast, the turnover of aggrecan is relatively fast compared to that of collagen. Aggrecan monomers form aggregates in cartilage by interaction of their N-terminal globular hyaluronan binding (G1)-domain with hyaluronan.[131,176] During aging, degradation products of aggrecan (C-terminal fragments) diffuse out of the tissue, while the remaining hyaluronan-bound N-terminal fragments accumulate with advancing age (Figure 1).[131,204] As a result, aggrecan subfractions can be obtained that contain mole-

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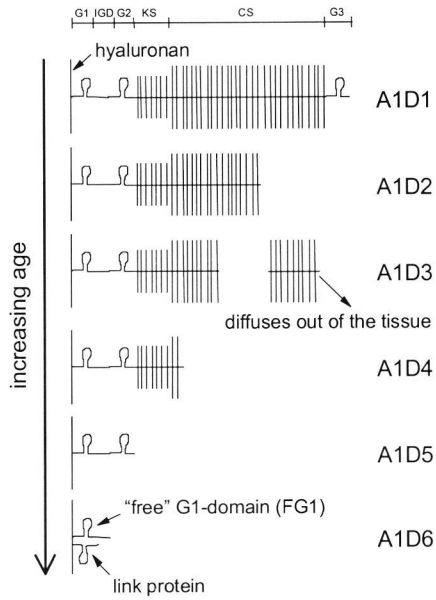


FIGURE 1 - Turnover of aggrecan monomer. Degradation of the aggrecan monomer with age results in loss of C-terminal fragments from the tissue and accumulation of the smaller fragments that remain bound to hyaluronan through their G1-domain. This results in aggrecan subfractions that contain molecules of decreasing size (A1D1 to A1D6), corresponding to aggrecan pools with different turnover rates *i.e.* residence time. G1: globular hyaluronan binding domain, IGD: interglobular domain, G2: globular domain 2, KS: keratan sulfate-rich region, CS: chondroitin sulfate-rich region, G3: globular domain 3.

cules of different size (A1D1 to A1D6); the smaller the fragments the higher their residence time in the matrix (Figure 1).[204] The half-life of the aggrecan subfractions in healthy adult human cartilage has been estimated to range from 3 to 24 years:[204] the initial processing of the intact monomer (A1D1) to a smaller molecule has a half-life of 3 years, while the ultimate processing of aggrecan as a "free" G1-domain (FG1), *i.e.* the actual turnover of aggrecan, has a half-life of 24 years.

The goal of the present study was to investigate pentosidine levels in cartilage aggrecan and collagen in relation to their respective rates of protein turnover. Pentosidine levels in aggrecan subfractions were used, together with biochemical data, to arrive at a quantitative measure of aggrecan turnover. For these calculations a theoretical model was used analogous to that used for determining the turnover rate of human articular cartilage aggrecan based on aspartic acid racemization data.[204] Furthermore, pentosidine levels in aggrecan isolated from osteoarthritic (OA) cartilage, in which the turnover of aggrecan has often been reported as increased,[154,178,182,200,324] were compared to those in normal cartilage. Estimations of the protein residence time were obtained from the racemization of aspartic acid.[204,341] This measure of protein residence time is based on the relatively fast racemization of aspartic acid from the L-form, in which it is built into proteins, into the D-form during residence in the tissue.[135,210]

EXPERIMENTAL PROCEDURES

Cartilage

Macroscopically normal human articular cartilage was obtained *post mortem* from femoral condyles within 18 hours after death from patients who had no known clinical history of joint disorders (age 7 to 103 years, n=50). For proteoglycan extraction and aggrecan purification, normal cartilage was obtained from knee joints surgically resected for bone tumors not involving the joint (age 9 to 93 years, n=25). OA cartilage was obtained from knee and hip joints at the time of joint replacement (age 39 to 77 years, n=11). All tissue samples were stored at -20°C until analyzed.

Purification of collagen

Articular cartilage collagen was purified by depleting the tissue of all proteoglycans and other non-collagenous proteins by sequential enzymatic treatment with chondroitinase ABC (Sigma, St. Louis, MO, U.S.A.) trypsin (Boehringer Mannheim, Mannheim, Germany), and *Streptomyces* hyaluronidase (Sigma) at 37°C as described by Schmidt *et al.*[289] This procedure resulted in the removal of more than 97% of the glycosaminoglycans with a minimal loss of collagen (< 1%, measured as hydroxyproline).

Proteoglycan extraction and preparation of aggrecan subfractions

Proteoglycans were extracted from full-depth cartilage, cryosectioned in 20 µm slices, with 4 M GuHCl (pH 7.0) at 4°C for 24 hours.[38] Aggrecan aggregates (A1 preparation) were purified from the dialyzed extract by associative CsCl equilibrium density gradient centrifugation.[40]

The aggregates in the A1 preparation of a 55-year-old donor were dissociated with 4 M GuHCl and subsequently fractionated into aggrecan monomer subfractions of decreasing buoyant density and decreasing molecular weight (A1D1 to A1D6).[204] "Free" G1-domain (FG1) was prepared from the A1D6 fraction by size exclusion high-performance liquid chromatography (HPLC).[51]

Pentosidine and amino acid analysis

Pentosidine and amino acid content were determined by HPLC after acid hydrolysis as described previously.[27,28] In short, samples were hydrolyzed in 6 M HCl at 110°C for 20-24 hours. After drying, the samples were dissolved in internal standard solution containing 10 µM pyridoxine (Sigma) and 2.4 mM homoarginine (Sigma). For pentosidine analysis, samples were diluted five-fold with 0.5% (v/v) heptafluorobutyric acid (Fluka, Buchs, Switzerland) in 10% (v/v) acetonitrile (Rathburn, Walkerburn, U.K.) and analyzed by HPLC.[27] Pentosidine analysis was standardized using the calibrated pentosidine solution kindly provided by Professor J.W. Baynes (University of South Carolina, Columbia, SC, U.S.A.). An aliquot of the samples used for pentosidine analysis was diluted 50-fold with 0.1 M sodium borate buffer (pH 11.4) and derivatized with 9-fluorenylmethyl chloroformate (Fluka) for HPLC amino acid analysis.[28]

The pentosidine content of the collagen and aggrecan samples is expressed either as nmol per gram of protein (assuming a molar weight of 300 kD for a triple helical collagen molecule[27] and 264 kD for an aggrecan core protein[88]) or as µmol per mol of lysine or arginine residues.

Percentage D-Asp in proteoglycan preparations

The percentage D-Asp (%D-Asp) in aggrecan subfractions (A1D1 to A1D6, and FG1) is taken from our previous work.[204] In A1 preparations from normal and OA cartilage, %D-Asp was determined by HPLC as described previously.[341] In short, samples were hydrolyzed in 6 M HCl at 100°C for 6 hours. After drying, the hydrolysates were dissolved in 0.1 M sodium borate buffer (pH 9.5) and derivatized with *o*-pthaldialdehyde/*N*-acetyl-L-cysteine (Sigma) for HPLC analysis.[341] All of the %D-Asp data were corrected for racemization during the hydrolysis step by subtracting the %D-Asp measured in the A1 preparation purified from young pig laryngeal cartilage (approximately 6 months old).[204]

Statistical analysis

Analysis of covariance (ANCOVA) and linear regression analysis were performed on data from mature subjects only (> 20 years) using SPSS version 8.0 for Windows (SPSS, Chicago, IL, U.S.A.); $p < 0.05$ was considered to represent statistically significant differences.

RESULTS

Age-related changes in pentosidine levels in cartilage proteins

After maturity (> 20 years), pentosidine accumulates linearly with age in human articular cartilage collagen ($r = 0.93$, $p < 0.0001$; Figure 2).[25,331,340] In a way that is qualitatively comparable to collagen, pentosidine levels accumulate with age in A1 preparations of cartilage aggrecan ($r = 0.82$, $p < 0.0001$; Figure 2). Nevertheless, over the entire age range pentosidine levels increased only 5-fold per mg aggrecan protein, compared to a 15-fold increase per mg collagen (rate of accumulation 0.11 vs. 0.32 nmol/gram protein/year, respectively; ANCOVA with age as covariate $p < 0.0001$). This 3-fold difference in accumulation rate could not be accounted for by differences in lysine or arginine content since collagen and aggrecan contain similar amounts of lysine (1.5-2%)

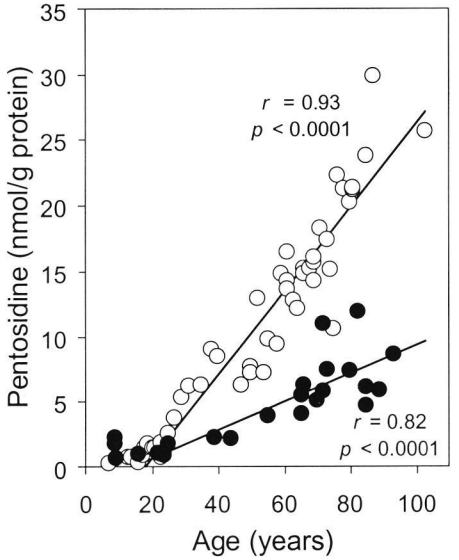


FIGURE 2 - Rates of pentosidine accumulation in articular cartilage aggrecan and collagen as a function of age. Pentosidine levels (nmol/gram protein) increase with age in aggrecan A1 preparations (●; 25 subjects, 9 - 93 years) and in collagen (○; 50 subjects, 7 - 103 years) isolated from normal human articular cartilage. A significant difference in pentosidine accumulation was observed between aggrecan and collagen ($p < 0.0001$).

and arginine (5-6%).[23,28,88] Hence, the rate of pentosidine accumulation expressed per lysine or arginine residue was also 3-fold lower in aggrecan than in collagen (0.37 vs. 1.40 µmol pentosidine/mol Lys/year and 0.21 vs. 0.64 µmol pentosidine/mol Arg/year, respectively; ANCOVA with age as covariate $p < 0.0001$ for both; data not shown). Based on the known proportions of collagen, aggrecan and other proteins in human articular cartilage,[205] it was calculated that in healthy adult cartilage more than 80% of the total pentosidine in cartilage was present in collagen (84.4% at age 70) and less than 10% was present in aggrecan (9.0% at age 70). This result was corroborated by pentosidine analyses of articular cartilage from which all proteoglycans and other non-collagenous proteins had been enzymatically removed: 82.1 ± 8.4% (n=50) of the total pentosidine in cartilage resided in collagen.

TABLE 1 Pentosidine in 55-year-old aggrecan

Fraction	Pentosidine/protein (nmol/gram)
A1	3.92
A1D1	1.06
A1D2	2.01
A1D3	3.19
A1D4	4.52
A1D5	6.51
A1D6	6.66
FG1	5.61

Pentosidine levels in aggrecan subfractions

The pentosidine levels for different buoyant density fractions of aggrecan (Figure 1), isolated from 55-year-old knee cartilage, are summarized in Table 1. Clearly, pentosidine levels increased from fraction A1D1 to A1D6, *i.e.* with decreasing size of the aggrecan molecules. Comparison of the pentosidine data with the extent of aspartic acid racemization (%D-Asp; previously published in Ref. 204) as a measure of protein residence time showed that pentosidine levels in the aggrecan subfractions increased with %D-Asp (Figure 3).

Since the G1-domain is relatively rich in arginine and lysine residues,[88] the increase in pentosidine concentration from A1D1 to A1D6 could also result from the greater concentration of the G1-domain, and therefore arginine and lysine residues, in the small molecules compared to the large molecules (Figure 1). However, also when expressed per arginine or lysine residue pentosidine levels increased from A1D1 to A1D6 (3.1 to 11.7 µmol/mol Arg and 4.5 to 18.0 µmol/mol Lys, respectively).

Pentosidine levels in aggrecan from normal and osteoarthritic cartilage

Pentosidine levels in A1 preparations from OA cartilage were significantly lower than in A1 preparations from normal cartilage (Fig-

ure 4A; ANCOVA with age as covariate $p < 0.001$), suggesting that aggrecan turnover is increased in OA cartilage. This was supported by the lower residence time (%D-Asp) measured for OA cartilage aggrecan (Figure 4B; ANCOVA with age as covariate $p < 0.05$). Thus, both pentosidine levels and %D-Asp of aggrecan from OA cartilage were low compared to aggrecan from normal cartilage (Figure 4 and 5). Interestingly, the relation between pentosidine levels and %D-Asp in A1 preparations from normal and OA cartilage of 16 donors (Figure 5) fits the same curve as the subfractions (A1D1 to A1D6) from the 55-year-old donor (presented in gray in Figure 5).

DISCUSSION

After maturity, pentosidine accumulates in aggrecan in articular cartilage in a way that is qualitatively comparable to collagen. The rate of pentosidine accumulation in aggrecan is 3-fold lower than that in collagen. A quan-

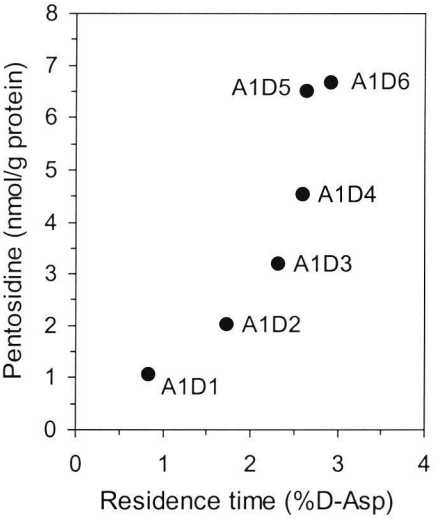
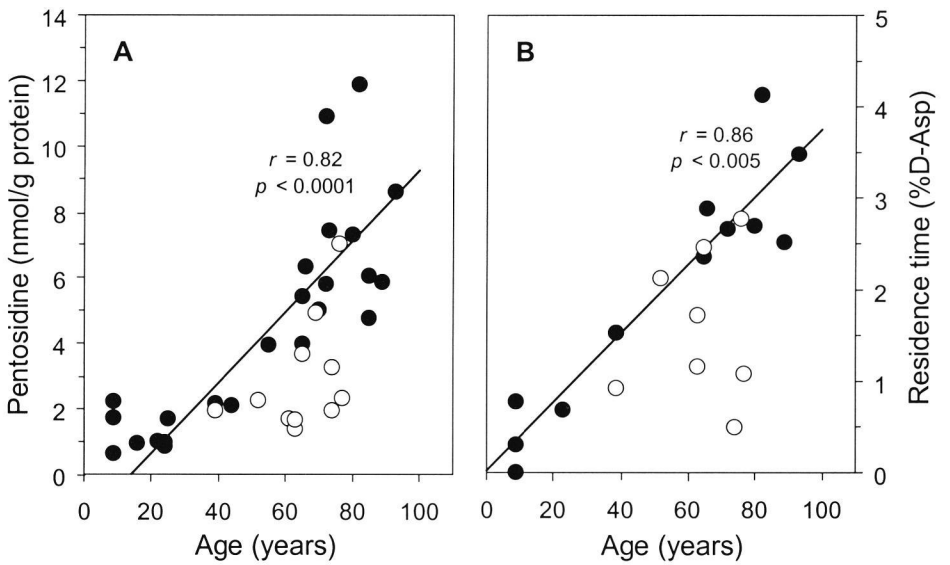


FIGURE 3 - Relationship of pentosidine levels with %D-Asp in aggrecan subfractions. Pentosidine levels (nmol/gram protein) increase with %D-Asp in aggrecan fractions containing molecules of different size (A1D1 to A1D6). The fractions were purified from knee cartilage of a 55-year-old donor.

FIGURE 4 - Pentosidine levels and %D-Asp in A1 preparations from human normal and osteoarthritic (OA) articular cartilage as a function of age. (**A**) Pentosidine levels (nmol/gram protein) in A1 preparations of normal (●; 25 subjects, 9 - 93 years) and OA (○; 11 subjects, 39 - 77 years) human articular cartilage. A significant difference in pentosidine content was observed between aggrecan from normal and OA cartilage ($p < 0.001$). (**B**) %D-Asp in A1 preparations of normal (●; 12 subjects, 9 - 93 years) and OA (○; 8 subjects, 39 - 77 years) human articular cartilage. A significant difference in %D-Asp was observed between aggrecan from normal and OA cartilage ($p < 0.05$).



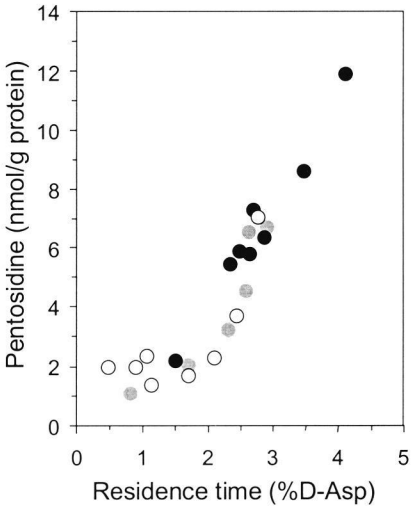


FIGURE 5 - Relationship of pentosidine levels with %D-Asp in A1 preparations. Pentosidine levels (nmol/gram protein) increase with %D-Asp in A1 preparations of normal (●; 8 subjects, 39 - 93 years, mean age 73.3 ± 17.1 years) and OA (○; 8 subjects, 39 - 77 years, mean age 63.6 ± 13.0 years) human articular cartilage. The results obtained for A1D1-A1D6 subfractions (Figure 3) are shown in gray.

titative comparison of these accumulation rates is only valid if pentosidine formation proceeds at similar rates in aggrecan and collagen. We have calculated pentosidine formation rates in both proteins based on data available from literature and from our own work; these calculations are presented in part A of the Appendix. *In vivo* pentosidine formation rates in aggrecan and collagen appeared to be very similar to one another: the calculated rate constants, k_F, for pentosidine formation in aggrecan and collagen are 2.22 and 1.99 µmol pentosidine per mol lysine per year, respectively (see Appendix part A). Therefore, the lower rate of pentosidine accumulation in aggrecan than in collagen most likely results from the higher rate of turnover of aggrecan compared to collagen.[208]

Pentosidine measurements in aggrecan fractions that contain molecules of different size (A1D1 to A1D6; Figure 1), revealed that pentosidine levels increase with increasing residence time of the protein (measured as aspartic acid racemization; %D-Asp[204]). Interestingly, the relationship between pentosidine levels and %D-Asp in A1 preparations was very similar to that of the aggrecan subfractions (Figure 5). The fact that these different approaches yielded similar results supports the argument that turnover is an important determinant of AGE accumulation.

The quantitative relation between pentosidine accumulation and protein turnover was studied within one protein, *i.e.* aggrecan, by using experimental pentosidine values to calculate the rate of turnover of the G1-domain ($k_{T(G1)}$). In doing this, we assumed that pentosidine per lysine increases linearly with age in the aggrecan G1-domain (as we showed for aggrecan A1 preparations) and that pentosidine formation occurs with similar kinetics throughout the aggrecan core protein. From our calculations in part B of the Appendix, we obtained $k_{T(G1)}$= 0.0355 per year, which is fairly close to our previous value of 0.0287 that was based on %D-Asp levels.[204] The present value of $k_{T(G1)}$ corresponds to a half-life for the aggrecan G1-domain of 19.5 years as compared with the previously determined value of 23.5 years[204] (see Appendix part B). Thus, with the assumptions made, the data suggest that pentosidine concentrations in the aggrecan subfractions, together with quantitative biochemical data, can be used as a quantitative measure to calculate the turnover rate of aggrecan G1. Since each aggrecan monomer is considered to be ultimately processed as its "free" G1, the calculated turnover equals the actual turnover of aggrecan itself.

From our data on aggrecan subfractions it seems that pentosidine and %D-Asp levels are equally good measures of protein residence time. However, when different types of protein are compared, it depends on the similarity of the formation rate of pentosidine or D-Asp between the proteins as to which of the parameters will be preferred. As an example, pentosidine formation rates in aggrecan and collagen are similar (see Appendix part A), while the rate of aspartic acid racemization in aggrecan is much higher

than that in collagen.[204,208] Thus, in the comparison of cartilage aggrecan and collagen, pentosidine levels can be readily used as a measure of protein residence time, while %D-Asp levels can only be used when the difference in racemization rates is taken into account.

Since it is generally accepted that AGE levels increase with age in long-lived proteins, it was at first sight surprising that no age-related increase in pentosidine levels was found in human aggrecan by Pokharna *et al.*;[264] their purified A1D1 fractions predominantly contained full-size, relatively "young" aggrecan monomers, irrespective of the age of the donor.[131,204] In the present study, we show a clear age-related accumulation of pentosidine in human articular cartilage aggrecan in A1 preparations that contain a mixture of "young" and "old" aggrecan species. The difference between Pokharna's A1D1 and our A1 preparations in pentosidine accumulation with age is consistent with data on aspartic acid racemization: the A1D1 fractions also failed to show an age-related increase in %D-Asp, while a clear age-related increase in %D-Asp is observed in the A1 preparations.[204] Thus, our study explains the apparent lack of pentosidine accumulation in aggrecan as reported by Pokharna *et al.*[264] and clearly shows that studies of separate molecular entities lead to different conclusions from studies performed on mixtures of different species.

Our data on both pentosidine levels and %D-Asp in aggrecan A1 preparations from OA and normal cartilage show that the residence time of the aggrecan molecules in OA cartilage is significantly lower than that in normal cartilage, *i.e.* OA cartilage aggrecan is "younger". This could be due either to increased synthesis of the intact aggrecan monomer (A1D1) in OA cartilage[37,200] or to an increased rate of removal of small aggrecan fragments, FG1 and link protein, from the tissue.[178] Our present results are inconclusive on the relative contribution of these processes to the increased aggrecan turnover during the OA disease process.

In summary, in the present study we have confirmed that protein turnover is a major determinant of AGE levels by comparing pentosidine levels in the different protein constituents of human articular cartilage, *i.e.* aggrecan and collagen. Our results suggest that the increase in pentosidine levels with protein residence time can be used as a quantitative measure of turnover of long-lived proteins, with advantages over aspartic acid racemization when cartilage collagen and aggrecan are compared. As illustrated by changes in pentosidine levels in OA cartilage, pentosidine concentrations in proteins from healthy and diseased tissues can be used to obtain more insight into the turnover changes that occur during disease processes.

ACKNOWLEDGEMENTS

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APPENDIX

Part A: Rate constant of pentosidine formation in collagen and aggrecan

The rate constant of pentosidine formation in collagen was calculated using our experimental data on the age-related increase in pentosidine in collagen (from Figure 2: pentosidine (nmol/gram protein) = 0.3189 x age (years) - 5.7913) and a mass balance equating the rate of accumulation of pentosidine in collagen with age to the difference between the rates of pentosidine formation and protein turnover (*i.e.* pentosidine removal). The increase in pentosidine relative to the amount of lysine is linear with age (data not shown). We have taken lysine concentrations in cartilage collagen from our experimental findings: the lysine concentration in collagen type II is 2.0×10^{-4} mol per gram protein (60 residues per 300 kD protein).

Using the mass balance on pentosidine in collagen and the fact that in collagen there is no change in lysine content with age ($p = 0.58$, data not shown), we have:

$$[Lys]_{COL}d[pentosidine/Lys]_{COL}/dt = k_{F(COL)}[Lys]_{COL} - k_{T(COL)}[Lys]_{COL}[pentosidine/Lys]_{COL} \quad [1]$$

where $[Lys]_{COL}$ is the concentration of lysine in collagen type II, in mol per gram collagen; $[pentosidine]_{COL}$ is the concentration of pentosidine in collagen in mol per gram protein at age 55 (from Figure 2: $[pentosidine]_{55} = 11.7 \times 10^{-9}$ mol per gram protein); $k_{F(COL)}$ is the rate constant of pentosidine formation in collagen in mol per mol lysine per year; $k_{T(COL)}$ is the turnover constant for collagen ($years^{-1}$), and t is age (years). Taking $k_{T(COL)}$ to be equal to 6.7×10^{-3} per year (based on %D-Asp levels)[341] and the linear increase in pentosidine per year in collagen to be 0.32×10^{-9} mol per gram protein (from Figure 2), we have:

$$\begin{aligned} k_{F(COL)} &= d[pentosidine/Lys]_{COL}/dt + k_{T(COL)}[pentosidine/Lys]_{COL} \quad [2] \\ &= (0.32 \times 10^{-9} / 2.0 \times 10^{-4}) + (6.7 \times 10^{-3} \times 11.7 \times 10^{-9} / 2.0 \times 10^{-4}) \\ &= 1.99 \text{ µmol pentosidine per mol Lys per year} \end{aligned}$$

Using a similar approach as for collagen, we calculated the rate constant of pentosidine formation in aggrecan. The calculation is based on a mass balance on the large monomer (A1D1), equating the rate of accumulation of pentosidine with age in this species to the difference between the rates of pentosidine formation and protein turnover. As was shown by Pokharna *et al.*,[264] pentosidine does not accumulate in the A1D1 fraction with age, resulting in a mass balance on aggrecan A1D1 that directly equates the rate of pentosidine formation to the rate of pentosidine removal through protein turnover. We have taken the lysine concentration in A1D1 from Doege *et al.*:[88] the lysine concentration in the A1D1 fraction is 1.04×10^{-4} mol per gram protein (28 residues per 268 kD protein).

Using the mass balance on pentosidine in A1D1 and the fact that in this fraction there is no pentosidine accumulation with age,[264] we have:

$$\begin{aligned} d([Lys]_{A1D1}[pentosidine/Lys]_{A1D1})/dt &= k_F[Lys]_{A1D1} - k_{T(A1D1)}[Lys]_{A1D1}[pentosidine/Lys]_{A1D1} \quad [3] \\ &= 0 \end{aligned}$$

where $[Lys]_{A1D1}$ is the concentration of lysine in the A1D1 fraction, in mol per gram A1D1; $[pentosidine/Lys]_{A1D1}$ is the concentration of pentosidine in the A1D1 fraction in mol per mol lysine; k_F is the rate constant of pentosidine formation in aggrecan in mol per mol lysine per year; $k_{T(A1D1)}$ is the turnover constant for the large monomer to undergo initial processing to a smaller molecule ($years^{-1}$), and t is age (years). Taking $k_{T(A1D1)}$ from our previous study[204] to be equal to 0.218 per year and the pentosidine level in A1D1 to be 1.06×10^{-9} mol per gram protein (Table 1), we have:

$$\begin{aligned} k_F = k_{T(A1D1)}[pentosidine/Lys]_{A1D1} &= 0.218 \times (1.06 \times 10^{-9} / 1.04 \times 10^{-4}) \quad [4] \\ &= 2.22 \text{ µmol pentosidine per mol Lys per year} \end{aligned}$$

Part B: The use of pentosidine levels as a quantitative measure of aggrecan turnover

We have employed a similar approach as in our previous study[204] to investigate whether pentosidine levels in different buoyant density fractions of aggrecan of one donor can be used as a quantitative measure of turnover, in a similar way to that based on aspartic acid racemization.

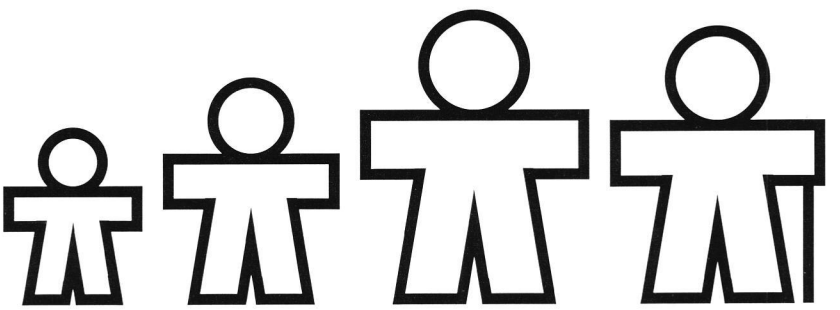
The model is based on mass balances on the large monomer (A1D1; see Appendix A) and the G1-domain, equating the rate of accumulation of pentosidine with age in these species to the difference between the rates of pentosidine formation and protein turnover. The turnover constants and changes in composition with age are taken from our previous work.[204] Because the pentosidine level in the lowest buoyant density fraction of aggrecan (A1D4) that could be isolated from the A1 preparation of a 22-year-old donor was similar to that in the A1 preparation and in the A1D1 (all ~1 nmol/gram protein; data not shown), we have taken the pentosidine level in the aggrecan G1-domain at age 20-25 to equal 1 nmol/gram protein. The formation constant, k_F, of pentosidine per mol lysine is assumed to be the same in the G1-domain as in the remainder of the monomer. The increase in pentosidine relative to the amount of lysine in the G1-domain is taken to be linear with age, as is observed for the aggrecan A1 preparations (data not shown). The lysine concentration in the G1-domain is taken from Doege *et al.*:[88] 2.19×10^{-4} mol of Lys per gram protein (8 residues per 36 kD protein).

To calculate the rate of G1 turnover ($k_{T(G1)}$) we used the mass balance on pentosidine in G1:

$$d([Lys]_{55yr}[pentosidine/Lys]_{55yr})/dt = k_F[Lys]_{55yr} - k_{T(G1)}[Lys]_{55yr}[pentosidine/Lys]_{55yr} \quad [5]$$

where $[Lys]_{55yr}$ is the concentration of lysine in the FG1 at age 55 years, in mol per gram of wet cartilage; $[pentosidine/Lys]_{55yr}$ is the concentration of pentosidine in the FG1 at age 55 years in mol per mol lysine, and $k_{T(G1)}$ is the turnover constant for the ultimate processing of the FG1, *i.e.* the actual turnover of aggrecan measured as turnover of its G1-domain ($years^{-1}$).

To calculate $d[Lys]_{55yr}/dt$ at age 55, a value for $d[FG1]/dt_{55yr}$ of 1.83 nmol per gram cartilage per year was used.[204] A concentration of 107 nmol FG1 per gram of wet cartilage at age 55 years[204] was used to calculate $[Lys]_{55yr}$. Unfortunately, the amount of FG1 material of the 55-year-old donor available for pentosidine analysis was limited, causing the pentosidine peak to approach the detection limit of the HPLC. Inasmuch as a reliable pentosidine number for FG1 is essential for the proposed calculations, the mean value for isolated FG1 and A1D5 (very similar to FG1) was used (Table 1), viz. 6.05 nmol per gram of protein. Inasmuch as A1D6 contains not only FG1 but also a substantial amount of link protein, the pentosidine value of this fraction was excluded from the calculations. Using this pentosidine value for FG1, the value of k_F as determined in Eq. [4] and a mass balance for FG1 as in Eq. [5], we obtain $k_{T(G1)} = 0.0355$ per year which, considering the assumptions made, is not inconsistent with the previously determined value of 0.0287 that was based on %D-Asp levels.[204] The present value of $k_{T(G1)}$ corresponds to a half-life for the aggrecan G1 of 19.5 years.



Chapter 5

Cross-Linking by Advanced Glycation Endproducts Increases the Stiffness of the Collagen Network in Human Articular Cartilage; a Possible Mechanism Through Which Age is a Risk Factor for Osteoarthritis

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Objective. *Age is an important risk factor for osteoarthritis. During aging, nonenzymatic glycation results in the accumulation of advanced glycation endproducts (AGEs) in cartilage collagen. We studied the effect of AGE cross-linking on the stiffness of the collagen network in human articular cartilage.*

Methods. *To increase AGE levels, human adult articular cartilage was incubated with threose. The stiffness of the collagen network was measured as the instantaneous deformation (ID) of the cartilage and as the change in tensile stress in the collagen network as a function of hydration (osmotic stress technique). AGE levels in the collagen network were determined as: N^{ε}-(carboxy(m)ethyl)lysine, pentosidine, amino acid modification (loss of arginine and (hydroxy-)lysine), AGE fluorescence (360/460 nm), and digestibility by bacterial collagenase.*

Results. *Incubation of cartilage with threose resulted in a dose-dependent increase in AGEs and a concomitant decrease in ID ($r = -0.81$, $p < 0.001$; up to 40% decrease at 200 mM threose),* i.e. *increased stiffness, which was confirmed by results from the osmotic stress technique. The increased stiffness strongly correlated with AGE levels (*e.g. *AGE fluorescence $r = -0.81$, $p < 0.0001$). Co-incubation with arginine or lysine, glycation inhibitors, attenuated the threose-induced increase in stiffness ($p < 0.05$).*

Conclusion. *Increasing cartilage AGE cross-linking by* in vitro *incubation with threose resulted in increased stiffness of the collagen network. Increased stiffness by AGE cross-linking may contribute to the age-related failure of the collagen network in human articular cartilage to resist damage. Thus, the age-related accumulation of AGE cross-links presents a putative molecular mechanism whereby age is a predisposing factor for the development of osteoarthritis.*

Submitted for publication

Introduction

Osteoarthritis (OA) is a common chronic disabling disorder for which age is the single greatest risk factor.[78,105] Although age-related changes in articular cartilage are likely to play a role, the mechanism through which age increases the susceptibility for joint degeneration is largely unknown. Swelling of cartilage, which is proportional to the amount of damaged collagen,[31] is the initial event in cartilage degeneration,[202] indicating that the development of OA starts with failure of the cartilage collagen network. With increasing age, the stiffness of the collagen network in articular cartilage increases,[35,122] which may result in an age-related increase in susceptibility to mechanically induced damage. Indeed, the resistance of the collagen network in articular cartilage to fatigue damage decreases with increasing age.[111,350] We hypothesize that the age-related increase in stiffness of the cartilage collagen network results in a decreased resistance of this network to mechanical failure and consequently an increase in the risk to develop OA.

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The question that has yet to be answered is: what causes the stiffness of the articular cartilage collagen network to increase with age? Because of the exceptionally long half-life (>100 years) of collagen molecules in articular cartilage,[341] they are susceptible to the accumulation of endproducts of nonenzymatic glycation. Nonenzymatic glycation of proteins is initiated by the reaction of sugars with lysine and arginine residues in proteins and eventually leads to the formation and age-related accumulation of advanced glycation endproducts (AGEs) such as N^{ε}-(carboxymethyl)lysine (CML)[91] and N^{ε}-(carboxyethyl)lysine (CEL),[3] and cross-links like pentosidine,[92,295] methylglyoxal-lysine dimer (MOLD),[57] and threosidine.[269] In articular cartilage, relatively high levels of AGEs accumulate with increasing age.[319,331,340] In a preliminary experiment, generation of AGEs resulted in stiffening of the articular cartilage collagen network,[25] consistent with reports on other collagenous tissues.[22,304] Thus, accumulation of AGEs makes the collagen network more brittle, as was described for lens capsules[22] and bone (Catanese J, Bank RA, TeKoppele JM, Iverson EP, Yeh OC, Keaveny TM; submitted) and as such increase the susceptibility of cartilage collagen to mechanical failure. Thus, accumulation of AGEs could be a molecular mechanism that causes age to be a major predisposing factor for the development of OA.

Threose is a highly reactive carbohydrate that is formed by nonenzymatic degradation of ascorbic acid both *in vitro* and *in vivo*.[193,252] Among the ascorbic acid degradation products L-threose is the most abundant (approximately 20-25%) and the most reactive degradation product.[193,237,252] L-threose rapidly modifies lysine residues in proteins[185] leading to the formation of characteristic AGEs, including CML,[89] formyl threosyl pyrrole,[236] threosidine,[269] and pentosidine.[236]

The present study was designed to determine the effects of AGE cross-linking on the stiffness of the collagen network in articular cartilage. The collagen network stiffness was mainly measured as the instantaneous deformation (ID) of the tissue in unconfined compression.[224] These ID results were confirmed by the osmotic stress technique as described by Basser *et al.*[35] To prevent other age-related changes in articular cartilage from confounding the interpretation of these experiments, cartilage was glycated *in vitro* (using threose as a model compound). In the present study, not only the stiffness, but also the extent of amino acid modification and AGE cross-linking of the collagen network were determined. Stiffness measurements were correlated with AGE levels. Furthermore, to confirm that the effects of threose on mechanical properties of the collagen network were indeed the result of increased AGE cross-linking, we tested the effect of incubation of cartilage with threose in combination with lysine or arginine, both inhibitors of the glycation process.[219,297]

EXPERIMENTAL PROCEDURES

Tissue samples

Macroscopically normal human articular cartilage was obtained *post mortem* within 18 hours after death from patients who had no clinical history of joint disorders. Samples of full-thickness cartilage, excluding the underlying bone, were taken from the femoral condyles of 30-, 31- and 37-year-old donors and from the superior region of a femoral head of a 43-year-old donor. All tissue samples were stored at -20°C until analyzed.

Carbohydrate incubations

Healthy full-depth cylindrical cartilage samples (5.5 mm diameter) were punched from the joints and immersed in 0.15 M NaCl for 24 hours at 4°C. Prior to the incubations, fixed charge density (FCD; see below) was measured to distribute samples evenly among the incubation groups according to their FCD. Incubations with threose were carried out in PBS, pH 7.4, containing 25 mM EDTA (Spectrum, Laguna Hills, CA, U.S.A.) as proteinase inhibitor in two independent experiments. Cartilage samples of a 37- and a 43-year-old donor were incubated with 0 to 200 mM threose (Sigma, St. Louis,

MO, U.S.A.) and with 200 mM threose in combination with 20 mM L-lysine (Sigma) or L-arginine (Sigma). All incubations were performed for 6 days at 37°C; control samples were incubated in the same solution without threose. Two or three cartilage samples were used per condition. After the incubations, samples were washed for 24 hours in 0.15 M NaCl at 4°C with a change of the NaCl solution after 16 hours. Samples were subjected to unconfined compressive loading to determine the ID (see below) both before and after the incubations. Using a similar approach as for threose, cartilage samples of a 30- and a 31-year-old donor were incubated with methylglyoxal (0-20 mM) and ribose (0-200 mM; Sigma). Methylglyoxal was prepared freshly by the acid hydrolysis of 1,1-dimethoxyacetone (Sigma) and the preparation was purified by destillation.[214]

Fixed charge density

Fixed charge density (FCD) is defined as the concentration of negatively-charged groups in the tissue (meq/gram) and represents the glycosaminoglycan content.[337] The FCD was determined by means of the tracer cation method described elsewhere,[209] using 0.015 M NaCl labeled with ^{22}Na as the equilibrating solution.

Instantaneous deformation

As a measure of the stiffness of the collagen network the instantaneous deformation (ID) of the cartilage was determined. ID was measured by subjecting the full-depth samples to unconfined compressive step-loading in a custom-built apparatus.[224] During the experiment, the sample was immersed in 0.15 M NaCl at 4°C in a transparent glass cell. The top surface of the sample was compressed against a transparent rigid plunger and viewed with a microscope equipped with a photo camera. Prior to loading, a preload of approximately 0.03 MPa was applied to ensure contact between the surface of the sample and the plunger. The sample was then loaded so that the initial pressure on its top surface was 3.0 MPa. The ID, expressed as the percentage increase in area of the articular surface, was measured within 10 seconds after loading. The ID of the samples was measured before and after the incubations and data are presented as the ratio of the ID after incubation over that before incubation.

Since the proteoglycan content of cartilage modulates the tensile stresses in the collagen network, it plays an indirect role in determining the ID.[224] Addition of EDTA to the carbohydrate solutions was used to prevent matrix metalloproteinase- and aggrecanase-mediated proteoglycan loss from the tissue during the incubation, which was confirmed by FCD measurements before and after the incubation.

Osmotic stress technique

To corroborate the effect of AGE cross-linking on the stiffness of the cartilage collagen network, the tensile stress in the collagen network as a function of hydration was determined for control and threose-incubated cartilage samples.[35] Full depth cartilage plugs, obtained from the femoral head of a 30-year-old donor, were sliced on a freezing microtome (Leitz), 400 µm sections being removed from the cartilage surface as well as from the deep zone. The test specimens thus represented mainly "middle zone" cartilage. We chose to use sections from the middle zone because in normal cartilage the FCD is known to be relatively uniform in this region.[206] Samples were incubated in 0.15 M NaCl, containing 300 mM L-threose (Sigma), 25 mM EDTA (Spectrum), 5 units/ml penicillin (Sigma), and 5 µg/ml streptomycin (Sigma). The incubation was carried out for 5 days at 37°C. The osmotic stress technique was carried out on sets of control samples and threose-incubated samples taken from adjacent sites on the femoral head. The method involved incubation of samples enclosed in dialysis tubing (Spectrum) in calibrated, osmotically active polyethylene glycol (PEG 20 kDa; Fluka, Buchs, Switzerland) solutions. The tensile stress exerted by the collagen network, P_c, can be calculated from the 'balance of forces' at

equilibrium hydration.[35] At equilibrium hydration, P_c together with the externally applied osmotic stress, π_{PEG}, both of which tend to squeeze water out of the tissue, are balanced by the osmotic pressure of the cartilage proteoglycans, π_{PG}. The tensile stress in the collagen network, P_c, is plotted as a function of collagen network hydration (normalized tissue volume: $V_{normalized} = (V_{total} - V_{collagen})/V_{collagen}$) that was also obtained by the method given in Basser *et al.*[35] The larger the change in collagen tensile stress per change in hydration, *i.e.* the steeper the slope of the $P_c/V_{normalized}$ curve, the greater the tensile stiffness of the collagen network.

Determination of collagen AGE levels

The methods employed for analysis of cartilage collagen AGE levels have extensively been described in our previous work.[340] Articular cartilage collagen was purified by depleting the tissue of all non-collagenous proteins by sequential enzymatic treatment with chondroitinase ABC (Sigma), trypsin (Boehringer Mannheim, Mannheim, Germany), and *Streptomyces* hyaluronidase (Sigma).[340] For analysis of CML, CEL, and pentosidine, purified cartilage collagen was reduced with sodium borohydride (Sigma).[340] After acid hydrolysis, an aliquot of these samples was removed for pentosidine and amino acid analysis. CML, CEL, and lysine content of the collagen hydrolyzates were measured as their *N*-trifluoroacetyl methyl esters by isotope dilution selected ion monitoring gas chromatography - mass spectrometry (SIM-GC/MS), using deuterated internal standards.[3,91] The pentosidine and amino acid content of the hydrolyzates were determined by high-performance liquid chromatography (HPLC).[27,28] The pentosidine content of the collagen samples is expressed as mmol per mol collagen, while the CML, CEL, arginine (Arg), hydroxylysine (Hyl), and lysine (Lys) contents of the collagen samples are expressed as mol per mol collagen, assuming 300 hydroxyproline residues per triple-helical collagen molecule.[27] From the comparison of the sum of the Arg, Hyl, and Lys residues per collagen molecule in threose treated samples with control samples, the mean percentage of modification of these glycation sensitive amino acids was calculated, assuming 0% modification in the control samples.

AGE fluorescence (λ_{ex}=360 nm, λ_{em}=460 nm) in purified cartilage collagen was measured after digestion of the collagen with papain (from Papaya latex, Sigma).[340] Papain buffer was used as a blank. AGE fluorescence is expressed as relative fluorescence units and normalized to the hydroxyproline content of the digest, measured after acid hydrolysis (papain contributed <1% of the hydroxyproline in the digests).[79,313]

The susceptibility of purified cartilage collagen to collagenase digestion was measured following digestion with *Clostridium histolyticum* collagenase (CLS 2, Worthington, Freehold, NJ, U.S.A.).[340] After the incubation, the supernatant and remaining tissue were separated and the amount of collagen in both fractions was estimated by measuring the amount of hydroxyproline.[79,313]

Statistical analysis

Linear regression analyses and one-way analysis of variance (ANOVA) with post-hoc Tukey-HSD tests were performed with SPSS version 10.0 for Windows (SPSS, Chicago, IL, U.S.A.); p-values < 0.05 were considered to represent statistically significant differences. When no individual data are given, data are expressed as mean ± S.D.

RESULTS

Effect of threose on AGE levels in articular cartilage collagen

AGE levels were determined in collagen that was purified from cartilage samples that had been incubated with different concentrations of threose. Following incubation with threose, the levels of CML and CEL increased significantly (2.7- and 7.2-fold, respectively; Figure 1A), while only a slight increase in the collagen pentosidine concentration was observed at 200 mM threose (1.2-fold; data not shown). Besides the concentrations of these chemically well-characterized AGEs, more general measures

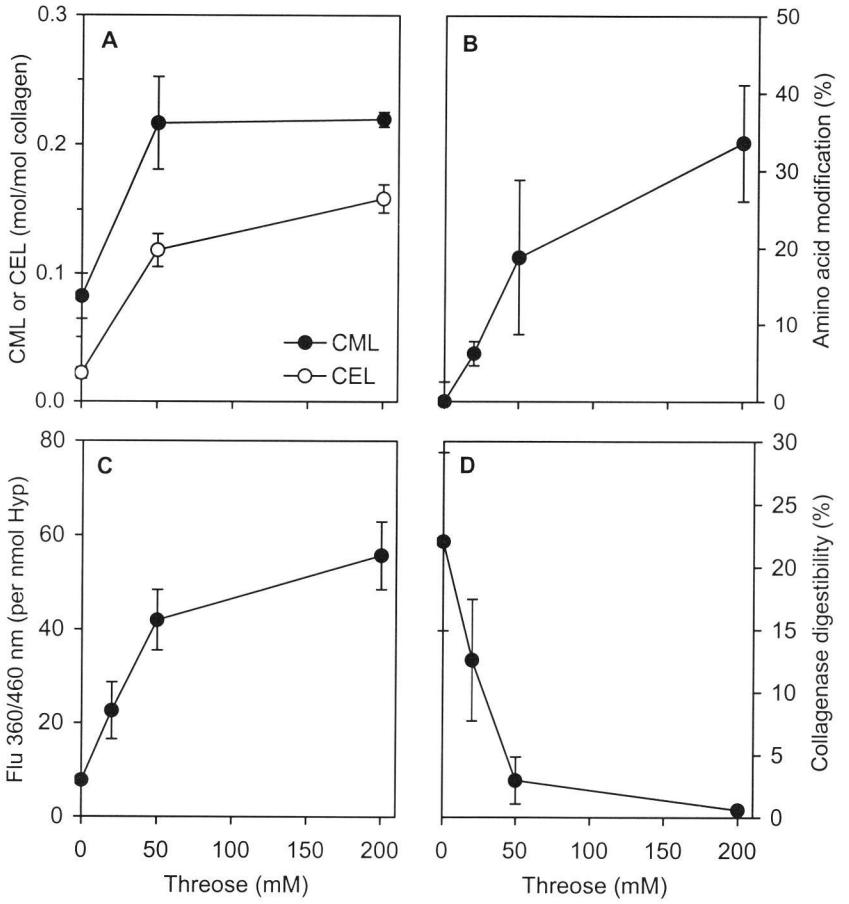


FIGURE 1 - Incubation with threose (6 days, 37°C) results in increased AGE levels in the collagen network in human articular cartilage. Data are mean (± S.D.) for measurement of CML and CEL levels (**A**), amino acid modification (**B**), AGE fluorescence (**C**), and collagenase digestibility (**D**) of the collagen network in at least 2 cartilage samples. Except for graph A, combined data of two independent experiments are shown.

of glycation and AGE cross-linking were determined in the threose incubated cartilage samples. Threose incubation resulted in extensive modification of Arg, Hyl, and Lys residues in cartilage collagen (17.7 ± 7.2, 57.2 ± 7.4, and 60.0 ± 10.7%, respectively at 200 mM of threose, n=2 independent experiments; the overall modification of Arg, Hyl, and Lys is shown in Figure 1B). The well-characterized AGEs (CML, CEL, and pentosidine) account for only a small fraction of all the AGEs formed during the incubation with threose *in vitro*: the total amount of CML, CEL and pentosidine represented only 1.13 ± 0.21% of the lysine modifications. AGE fluorescence, a general measure of cross-linking AGEs, was also increased in threose-incubated samples (Figure 1C). Consistently, threose reduced the digestibility of the collagen by bacterial collagenase, which represents a functional measure of cartilage collagen cross-linking (Figure 1D).

Except for pentosidine, all AGE measures (CML and CEL levels, overall amino acid modification, AGE fluorescence, and collagenase digestibility) correlated very well with one another ($r > 0.80$ in all cases).

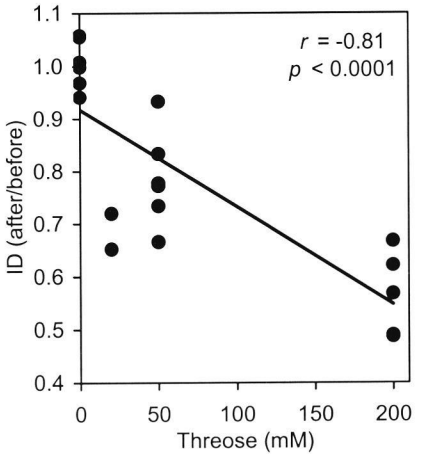


FIGURE 2 - Incubation with threose (6 days, 37°C) results in a concentration dependent decrease in ID of the collagen network, *i.e.* increase in stiffness. Data represent the ID after/before for each individual cartilage sample (combined results of two independent experiments).

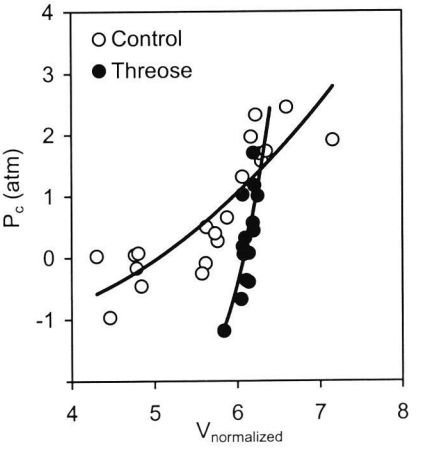


FIGURE 3 - Incubation with threose (300 mM, 5 days, 37°C) results in an increase in tensile stiffness of the collagen network in human articular cartilage. The collagen network tension, P_c, is plotted versus the normalized volume of the sample, $V_{normalized}$, for both control and threose-incubated samples. The graph after threose incubation has a steeper slope ($\Delta P_c/\Delta V_{normalized}$) than that of the control samples, indicating increased stiffness of the collagen network.

Effect of threose on the stiffness of the articular cartilage collagen network

The stiffness of the collagen network in the threose-incubated cartilage samples can be expressed as the ratio of the ID after incubation over the ID before incubation (ID after/before). If the incubation does not affect the stiffness of the collagen network, this ratio will equal 1. As expected, the ratio of the control samples was 1.00 ± 0.05 (Figure 2, incubation with 0 mM threose). A ratio < 1 reflects a decrease in deformation, and therefore an increase in stiffness of the collagen network due to the threose incubation. Clearly, incubation of cartilage with up to 200 mM of threose resulted in a dose-dependent increase in stiffness (r = -0.81, p < 0.0001; Figure 2). At 200 mM threose, the ID was decreased by 40%, reflecting a major increase in stiffness of the collagen network. Curves of the tensile stress in the collagen network (P_c) as a function of hydration ($V_{normalized}$; Figure 3) were obtained by the osmotic stress technique of Basser *et al.*[35] Incubation with threose resulted in a steeper slope of the $P_c/V_{normalized}$ curve, *i.e.* an increase in tensile stiffness of the collagen network in human articular cartilage. Increased AGE cross-linking of collagen in the threose-incubated cartilage samples was confirmed by analyses of AGE fluorescence, pentosidine, and amino acid modification. Consistent with the ID experiments, all AGE measures were increased in collagen purified from the threose-incubated samples compared to the control samples; pentosidine and AGE fluorescence increased 2.4- and 9-fold respectively, while the overall amino acid modification in the threose-incubated samples was 28.5 ± 1.5% (data not shown).

Correlation of collagen network stiffness with AGE levels

In the present study, we investigated the effect of *in vitro* glycation by threose on the stiffness of the cartilage collagen network, in combination with measurements of AGE levels. Because AGE levels were measured specifically in collagen (purified from cartilage by enzymatic removal of all non-collagenous proteins) and because stiffness

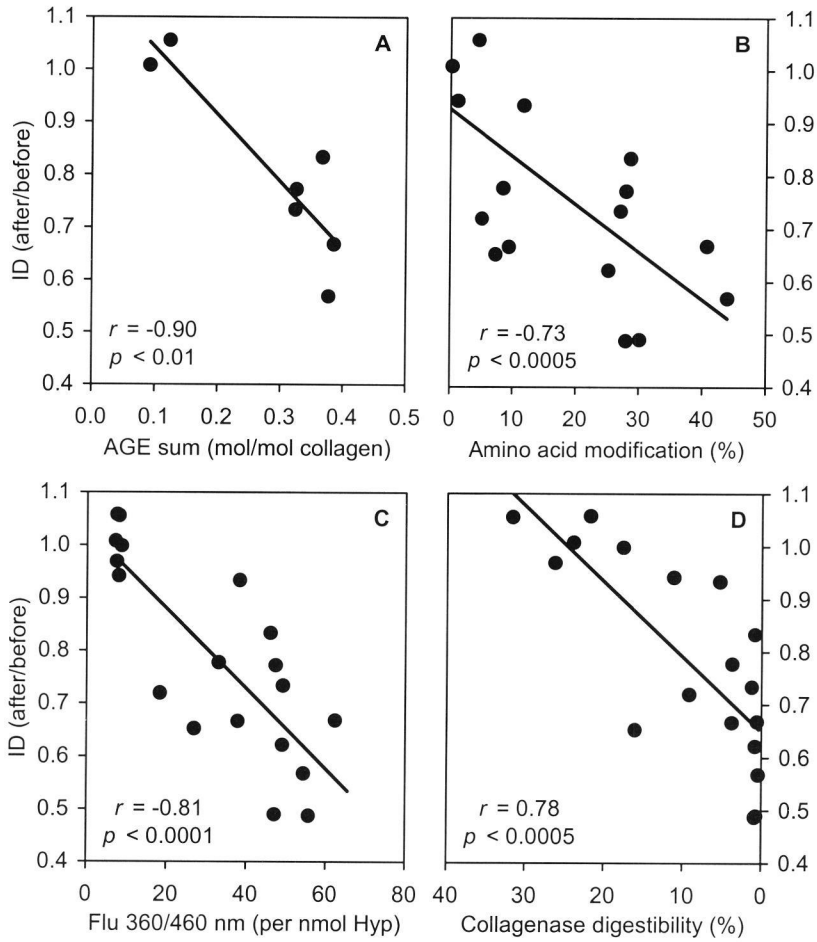


FIGURE 4 - The stiffness of the collagen network in articular cartilage is highly correlated with all AGE measures. Correlation of the ID after/before of cartilage samples incubated at different threose concentrations with the sum of CML, CEL and pentosidine (AGE sum; **A**), amino acid modification (**B**), AGE fluorescence (**C**), and collagenase digestibility (**D**). Except for graph A, combined data of two independent experiments are shown. Note that the X-axis of graph D has a reversed scale for a lower digestibility represents a higher degree of AGE cross-linking.

methods were used previously validated as measuring the stiffness of the collagen network,[35,224] stiffness measurements could be directly related to AGE levels.

The decrease in ID, *i.e.* the increase in stiffness, of the cartilage collagen network due to threose was strongly correlated with the collagen AGE levels. The decrease in ID correlated significantly with the sum of the well-characterized AGEs CML, CEL, and pentosidine in the collagen (r = -0.90, p < 0.01; Figure 4A). The ID also decreased with increasing overall amino acid modification and AGE fluorescence in the collagen (r = -0.73, p < 0.0005; Figure 4B and r = -0.81, p < 0.0001; Figure 4C, respectively). Furthermore, the threose-mediated increase in stiffness (decrease in ID) was related to a decrease in digestibility of the collagen by bacterial collagenase (indicative of increased collagen cross-linking; r = 0.78, p < 0.0005; Figure 4D).

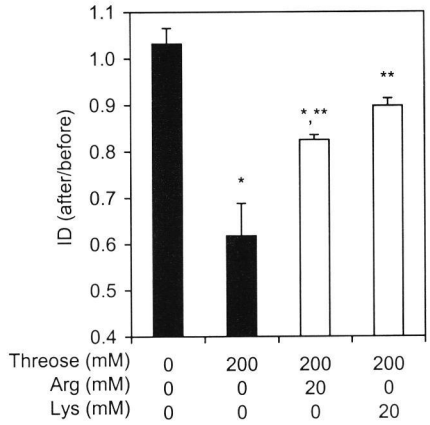
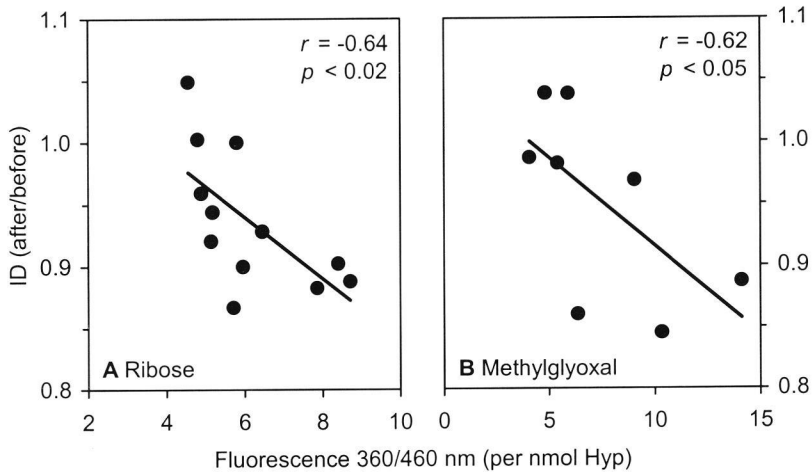


FIGURE 5 - Addition of Lys or Arg to the incubation with threose (6 days, 37°C) attenuates the threose-induced increase in stiffness of the cartilage collagen network. The mean (± S.D.) ID after/before is shown for at least 2 cartilage samples per condition. Cartilage was incubated with 0 (control) or 200 mM threose (black bars) and with 200 mM threose with the addition of 20 mM Arg or Lys (white bars). Asterisks indicate statistically significant differences compared to control (one asterisk; $p < 0.05$) or compared to 200 mM threose without additions (two asterisks; $p < 0.05$). The increase in collagen network stiffness by threose is glycation-specific because it can be inhibited by co-incubation with Arg or Lys.

Inhibition of the effect of threose on cartilage collagen stiffness

To confirm that the threose-induced increase in stiffness of the collagen network was indeed due to the increased AGE cross-linking, Arg or Lys were added to the threose incubations as inhibitors of the glycation and cross-linking process.[219,297] These amino acids were expected to compete with the cartilage proteins for the available threose, thereby preventing threose from forming AGE cross-links in the cartilage proteins. The highest threose concentration (200 mM) used in the present study resulted in a 40% decrease in ID (Figures 2 and 5), *i.e.* a substantial increase in stiffness of the cartilage collagen network. This decrease in ID by 200 mM threose could be inhibited by 50% in the presence of 20 mM Arg ($p < 0.05$ compared to 200 mM threose alone; Figure 5). As high as 68% inhibition was observed in the presence of 20 mM Lys ($p < 0.05$ compared to 200 mM threose alone; Figure 5). The resulting stiffness was unchanged from control levels ($p = 0.09$; Figure 5). The fact that Lys is a more potent inhibitor than Arg is consistent with the higher reactivity

FIGURE 6 - Correlation of the stiffness of the collagen network in articular cartilage with AGE levels, as observed after threose incubation (Figure 4), could be confirmed by incubation of human articular cartilage with ribose and methylglyoxal (6 days, 37°C). The ID after/before of cartilage samples incubated with different concentrations of ribose (up to 200 mM; **A**) and methylglyoxal (up to 20 mM; **B**) was significantly correlated with AGE fluorescence.



of threose towards Lys than towards Arg (this manuscript and Ref. 185).

Effect of other carbohydrates on the stiffness of the articular cartilage collagen network

Besides the effect of threose, the effects of ribose and methylglyoxal on the stiffness of the cartilage collagen network were investigated to see whether the results obtained with threose could be generalized to other carbohydrates. The increase in stiffness of cartilage collagen was significantly correlated with AGE fluorescence after ribose and methylglyoxal incubation (one-tailed regression analysis $r = -0.64$, $p < 0.02$ and $r = -0.62$, $p < 0.05$; Figure 6A and 6B, respectively). Thus, both the experiments with ribose and methylglyoxal confirm the relation between AGE levels and collagen network stiffness that is observed after threose incubation.

DISCUSSION

In the present study, we show that incubation of human articular cartilage with threose resulted in extensive AGE formation in the cartilage collagen network. Consistent with previous reports, CML and CEL were formed in substantially greater amounts than pentosidine.[89,236] Threose preferentially reacted with the amino-group of Hyl and Lys residues, and to a lesser extent with the guanidino-group of Arg residues. The observed 3-fold difference in reactivity between Hyl/Lys and Arg is consistent with results by Lee *et al.*[185] Also consistent with previous findings using other carbohydrates is that only 1.1% of the Lys loss in cartilage collagen in our incubations can be explained by formation of the well-characterized AGEs CML, CEL, and pentosidine.[3,57] In addition, our recent *in vivo* findings indicated that only 4.7% of the age-related loss of (hydroxy-)lysine residues in articular cartilage collagen can be explained by CML, CEL, and pentosidine.[340] Thus, these AGEs do not complete the mass balance and should only be considered as markers of overall AGE cross-linking. Other AGEs including formyl threosyl pyrrole, a major threose-derived AGE,[236] and threosidine, a Lys-Lys cross-link,[269] as well as MOLD and GOLD[57] were not measured in this study, but probably also contribute to threose mediated cross-linking of collagen. The cross-linking nature of the AGEs formed during the threose incubation was shown not only by the threose-induced AGE fluorescence,[236] but also by the decrease in digestibility of cartilage collagen by bacterial collagenase after threose incubation.[340]

Incubation of articular cartilage with threose clearly resulted in an increase in the stiffness of the collagen network, and this increased stiffness correlated very well with AGE levels measured in purified collagen. These results, based on measurements of ID, were confirmed by the osmotic stress technique. The correlation of stiffness with AGE levels is not unique to threose; similar correlations were found after incubation of cartilage with ribose, which results in the formation of AGE cross-links such as pentosidine[92,295] and the non-fluorescent NFC-1,[256] and methylglyoxal, which forms AGE cross-links such as MOLD.[57]

The addition of Lys or Arg to the incubation of cartilage collagen with threose profoundly reduced the effect of threose on the stiffness of the collagen network, as has been shown for rat tail tendons incubated with glucose.[219] Lys appeared to be a more potent inhibitor than Arg, which is consistent with the higher reactivity of threose with Lys than with Arg (this manuscript and Ref. 185). Thus, specifically interfering with the glycation process through the addition of Lys or Arg to the threose incubations, resulted in inhibition of stiffening of the collagen network. This indicates that the effect of threose on the stiffness of the collagen network is not only highly correlated with AGE levels, but also that it is indeed due to the AGE cross-linking.

In the present study, we used the ascorbic acid degradation product threose as a model glycating agent. As a consequence, our results may also contribute to our understanding of the role of ascorbic acid in

the development of OA.[212] The limited studies available suggest that ascorbic acid may have a protective effect on development of spontaneous OA in guinea pigs and progression of established OA in humans.[213,216] The potentially beneficial effect of ascorbic acid has been attributed to its antioxidant properties, the fact that it is a cofactor for collagen prolyl and lysyl hydroxylase,[172,234] and its ability to stimulate chondrocyte collagen and aggrecan synthesis,[80,234] which likely results from stimulation of collagen type II, aggrecan and prolyl hydroxylase gene and protein expression (Virginia Kraus, personal communication). The present results suggest that high levels of ascorbic acid may increase the susceptibility of the collagen network to degeneration through AGE cross-linking of collagen by threose, a product of ascorbic acid degradation. Pro-oxidant activities of ascorbic acid resulted in oxidation of articular cartilage collagen *in vitro* in explant cultures (Virginia Kraus, personal communication). Thus, ascorbic acid can potentially impact joint health by a variety of means. A trial is currently being conducted in a spontaneous *in vivo* OA model in guinea pigs, exploring the effects of high and moderate levels of ascorbic acid on the development of OA compared to a minimal, but nonscorbutic, level of the vitamin.

Based on our *in vitro* results we conclude that AGE cross-linking of articular cartilage collagen results in increased stiffness of the collagen network. Although AGE levels *in vivo* are lower than in our *in vitro* incubations, the age-related accumulation of AGE cross-links in collagen in human adult articular cartilage seems a plausible mechanism for the observed age-related increase in collagen network stiffness in human articular cartilage.[35,122] As a result of stiffening, accumulation of cross-linking AGEs makes the cartilage collagen network more brittle (Chen AC, Temple MM, Ng DM, Richardson CD, DeGroot J, Verzijl N, TeKoppele JM, Sah, RL; unpublished results). Increased brittleness may in turn contribute to the age-related failure of cartilage to resist mechanical damage, and thus be a factor that predisposes aged cartilage to damage and, eventually, the development of OA.

Furthermore, the inhibitory effect of Lys and Arg on the induction of stiffness in the collagen network through AGE cross-linking presents interesting therapeutic possibilities. According to our hypothesis and present data, agents that prevent AGE accumulation[52] as well as agents that are proposed to "break" already formed AGE cross-links[335,362] may, through prevention of an increase in collagen network stiffness, slow down the development of OA.

ACKNOWLEDGEMENTS

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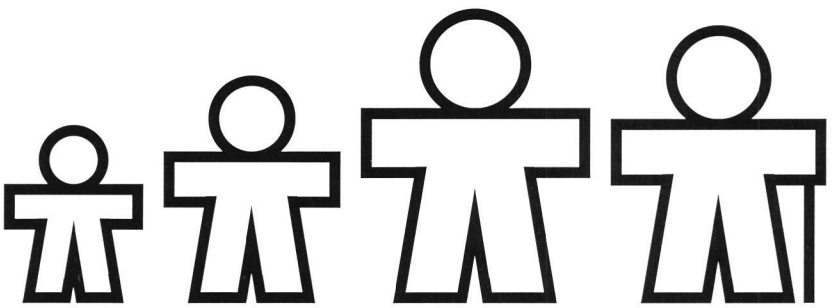

Chapter 6

Advanced Glycation Endproducts in Human Articular Cartilage Collagen Predispose to the Development of Osteoarthritis

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Objective. *The most prominent risk factor for osteoarthritis (OA) is age. The age-related accumulation of advanced glycation endproducts (AGEs) in cartilage is believed to contribute to the susceptibility to develop OA. We performed a cross-sectional study to compare cartilage collagen AGEs between OA-affected and -unaffected individuals.*

Methods. *Collagen turnover is known to influence AGE levels and is increased in early OA. Therefore, our study was strictly limited to macroscopically normal knee cartilage from donors with and without focal cartilage degeneration at autopsy (N_{DEG}: n=22, age 51-85 years, and control: n=23, age 46-83 years, respectively). Despite this limitation, collagen turnover was measured as the level of aspartic acid racemization. AGEs were measured as fluorescence at 360/460 nm, browning (absorption at 340 nm), and pentosidine levels.*

Results. *Unexpectedly, collagen turnover was increased in N_{DEG} cartilage compared with control cartilage ($p < 0.05$), which hampers proper comparison of absolute AGE levels. To compensate for the effects of turnover differences, AGE levels were corrected for collagen turnover. Corrected AGE levels,* i.e. *AGE formation rates, were higher in N_{DEG} cartilage than in control cartilage (fluorescence and browning: both $p < 0.01$; pentosidine: N.S.).*

Conclusion. *An increased rate of AGE formation in normal cartilage collagen of subjects with focal cartilage degeneration supports a role for AGE cross-linking as a risk factor for OA. Furthermore, our results of increased collagen turnover in N_{DEG} cartilage suggest that successful remodeling of the collagen network is possible in adult human articular cartilage. Whether or not this increased collagen turnover is a primary or secondary event in cartilage degeneration requires further investigation.*

SUBMITTED FOR PUBLICATION

Introduction

Osteoarthritis (OA) is the most common joint disorder in older subjects and contributes strongly to functional disability of the elderly. Although age is the main risk factor for OA,[78,102,105] the mechanisms underlying this relationship are largely unknown. Age-related structural changes in articular cartilage are potential factors in the susceptibility of cartilage to develop OA.[25,54,83,158]

A prominent feature of aging is the nonenzymatic modification of tissue proteins by reducing sugars. Nonenzymatic glycation is initiated by the reaction of a sugar with a protein lysine or arginine residue. This eventually leads to the formation of a variety of fluorophores and chromophores, collectively known as the advanced glycation endproducts (AGEs). Once formed, AGEs are not removed unless the protein is removed. Consistently, AGEs accumulate with age in long-lived proteins[90,295] at a rate that is largely determined by the rate of protein turnover.[341] Articular cartilage collagen has an exceptionally long half-life[341] which results in the accumulation of an abundant amount of AGEs such as pentosidine, a cross-link formed from lysine, a sugar, and arginine.[295,340] Besides this well-characterized AGE, general measures of AGE cross-linking like fluorescence at 360/460 nm and browning also increase with age in cartilage collagen.[340]

AGE cross-linking results in stiffening of the collagen network,[25] which - through increasing brittleness[22,68] - increases the susceptibility to damage. Furthermore, high AGE levels in cartilage impair the synthetic

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capacity of chondrocytes, which diminishes the ability of chondrocytes to restore matrix integrity after injury and may contribute to progression of cartilage defects into OA.[83] Based on these findings, age-related accumulation of AGE cross-links in cartilage collagen may well explain why age is a predisposing factor for the development of OA. Recently, *in vivo* proof of our hypothesis was obtained from a study in which AGE levels in articular cartilage in the knee joints of Beagle dogs were selectively enhanced by intra-articular ribose injections prior to induction of OA by anterior cruciate ligament transection.[265] Dogs with enhanced cartilage AGE levels showed an increased sensitivity to develop OA.[84] In humans, our hypothesis is not easily tested because the age-related increase in AGE levels occurs over a period of 20 to 60 years. Therefore, a cross-sectional study was designed using human articular cartilage obtained at autopsy. If indeed AGEs predispose to the development of OA, individuals with high cartilage collagen AGE levels should develop more severe OA (or earlier in life) than individuals with low AGE levels. Thus, cartilage collagen from individuals with OA should contain higher levels of AGE cross-links than that from age-matched healthy controls.

Because collagen AGE levels are largely determined by the rate of protein turnover[341] and because an increase in collagen turnover is an early feature of OA,[6,154,242,258] our hypothesis needed to be tested by measuring collagen AGE levels in normal cartilage from OA-affected and -unaffected individuals. Therefore, we chose to use visually intact cartilage from knees with focal, preclinical cartilage degeneration at autopsy (N_{DEG})[334] and compared its AGE levels with levels in cartilage from donors without any sign of cartilage degeneration (control). N_{DEG} cartilage has a normal proteoglycan content, synthesis and release, a low level of damaged collagen, and a low Mankin grade (0-1; which is comparable to control cartilage),[29,180,200] and therefore can be considered "normal". Notwithstanding, to account for the effect of possibly different rates of collagen turnover on AGE levels,[341] the residence time of collagen was also measured in the present study. As in our previous work, a good estimation of collagen residence time was obtained by measuring the percentage D-aspartic acid (%D-Asp).[341] This unique measure of protein residence time is based on the relatively fast racemization of aspartic acid from the L-form, in which it is built into proteins, into the D-form during the course of time.[135,210] In addition to AGE measures and collagen residence time, the enzymatic collagen modifications lysyl hydroxylation and pyridinoline cross-linking were determined, because these modifications may also affect the quality of the cartilage collagen network.[34]

Thus, the main focus of the present study was to compare AGE levels in normal (unaffected) cartilage from individuals with and without focal cartilage degeneration in order to address the question whether high AGE levels are associated with and therefore might predispose to OA. Because a difference in the residence time of the collagen network would bias possible differences in AGE levels the collagen residence time was determined as well.

EXPERIMENTAL PROCEDURES

Cartilage samples

Human articular cartilage was obtained *post mortem* from femoral condyles within 18 h after death. Donors had no known clinical history of joint disorders. Donors were selected either for knees with focal degenerative cartilage (*i.e.* affected donors with preclinical cartilage degeneration)[334] or for knees without any sign of degeneration (*i.e.* unaffected donors).[180] Slices of full-thickness cartilage, excluding the underlying bone, were cut from the weight-bearing areas (central parts) of the condyles of both knees. Macroscopically normal cartilage from knees with focal cartilage degeneration (N_{DEG}; Mankin grade 0-1) was compared to cartilage from knees without any sign of degeneration (control; Mankin grade 0-1).[180] Both N_{DEG} and control cartilage had a glossy, completely smooth surface and a healthy

appearance. Cartilage was used from a total of 22 donors with N_{DEG} cartilage and 23 age-matched controls; ages ranged from 46 to 85 years (mean age of N_{DEG} and control cartilage donors was 69.5 ± 2.0 and 66.3 ± 2.5 years, respectively; N.S.). For each donor, 3 cartilage samples were analyzed that were taken randomly from the weight-bearing areas of the condyles of both knees. It was demonstrated that the mean value of 3 random samples was representative of the entire joint for all parameters tested (data not shown). All tissue samples were stored at -20°C until analysis.

Purification of collagen

Articular cartilage collagen was purified by depleting the tissue of all proteoglycans and other non-collagenous proteins by sequential enzymatic treatment with chondroitinase ABC (Sigma, St. Louis, MO, U.S.A.), trypsin (Boehringer Mannheim, Mannheim, Germany), and *Streptomyces* hyaluronidase (Sigma) at 37°C.[289,341] From some of the N_{DEG} cartilage donors, degenerated cartilage (DEG; Mankin grade 4-8), identified macroscopically by its fibrillated surface, was analyzed as well.[180] In subsets of control samples (donors aged 62-83 years, n=7) and paired samples of N_{DEG} and DEG cartilage (donors aged 62-77 years, n=4) collagen loss during the enzyme treatments was monitored. For this purpose, supernatants from the subsequent enzymatic extractions were collected and hydrolyzed in 6 M HCl at 110°C for 20-24 h. The percentage collagen loss was estimated by measuring the amount of hydroxyproline in the hydrolyzates of all three supernatants and the remaining tissue using a colorimetric assay.[79,313]

Analytical procedures

Collagen-linked fluorescence (λ_{ex}=360 nm, λ_{em}=460 nm) and browning (absorption at 340 nm) were measured in papain digests of cartilage collagen (0.5-2 mg collagen in 250 µl of papain buffer) as described elsewhere.[340] One aliquot of the papain digests (100 µl) was hydrolyzed in 1 ml of 6 M HCl at 110°C for 20-24 h for cross-link and amino acid analysis, while another aliquot (25 µl) was hydrolyzed in 1 ml of 6 M HCl at 100°C for only 4 h for %D-Asp analysis (analyses are described below). Both fluorescence and browning were expressed in relative units and normalized to the hydroxyproline content of the digest that was obtained from amino acid analysis (papain contributed <1% of the hydroxyproline in the digests).

Collagen cross-link content and amino acid composition were determined by high-performance liquid chromatography (HPLC).[27,28] After drying, overnight (20-24 h) hydrolyzates of the papain digests were dissolved in water containing the internal standards pyridoxine (10 µM; Sigma) and homoarginine (2.4 mM; Sigma). For collagen cross-link analysis (hydroxylysylpyridinoline (HP) and pentosidine), samples were diluted five-fold with 0.5% (v/v) heptafluorobutyric acid (Fluka, Buchs, Switzerland) in 10% (v/v) acetonitrile (Rathburn, Walkerburn, U.K.) and analyzed by HPLC.[27] For amino acid analysis, an aliquot of the cross-link samples was diluted 20-fold with 0.1 M borate buffer (pH 11.4), derivatized with 9-fluorenylmethyl chloroformate (Fluka) and analyzed by HPLC.[28] Pentosidine, HP, and hydroxylysine (Hyl) contents of the collagen samples are expressed as mol per mol collagen, assuming 300 hydroxyproline (Hyp) residues per triple-helical collagen molecule.[27]

The percentage D-Asp (%D-Asp) in cartilage collagen was determined by HPLC in aliquots of the papain digests that had been briefly (4 h) hydrolyzed in 6 M HCl.[17,341] Dried hydrolyzates were dissolved in 1 ml of 0.1 M sodium borate buffer (pH 9.5). An aliquot of the samples (20 µl) was derivatized with 5 µl reagent containing *o*-phtaldialdehyde (30 mM; Sigma) and *N*-acetyl-L-cysteine (60 mM; Sigma) in 30% (v/v) methanol (Rathburn) in 0.1 M sodium borate buffer (pH 9.5) and analyzed by HPLC.[341] All of %D-Asp data were corrected for the amount of D- and L-Asp present in papain (10-20% of the aspartic acid in the samples) and for racemization during the hydrolysis step. With respect to the lat-

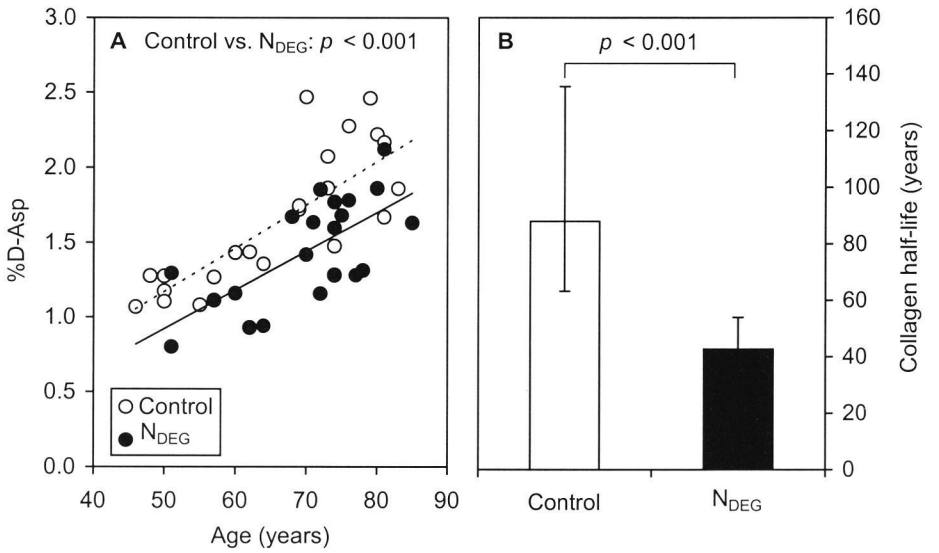

FIGURE 1 - A. Aspartic acid racemization (%D-Asp; a measure of collagen residence time) in collagen from control cartilage (open circles) and from normal cartilage from knees with focal degeneration (N_{DEG}; filled circles). Linear regression lines are shown for both control (dashed line; $r = 0.77$, $p < 0.001$) and N_{DEG} cartilage (solid line; $r = 0.70$, $p < 0.001$). **B.** Mean and 95% confidence interval of the half-lives of collagen from control and N_{DEG} cartilage. Collagen from N_{DEG} cartilage had a lower residence time - *i.e.* faster turnover - than collagen from control cartilage.

ter, papain digests of reference cartilage collagen samples (n=5, ages 2.5, 17, 28, 46, and 74 years) were included in each hydrolysis series. The intercept of the graph for %D-Asp versus donor age of these reference samples was subtracted from all individual %D-Asp values.[341]

Based on the %D-Asp data, half-lives of control and N_{DEG} cartilage collagen were calculated using a method similar to that employed in our previous work.[341] For each individual sample the %D-Asp per donor age was assessed, which represents the mean residence time of collagen in the particular cartilage sample over the entire life span of the donor. The mean (± SEM) value of each group was used to calculate the collagen half-life (mean ± 95% confidence interval) based on the model described by Maroudas *et al.*[208]

Statistical analysis

Statistical analyses were performed using SPSS software version 10.0 for Windows (SPSS, Chicago, IL, U.S.A.). Two-group comparisons were analyzed using Student's t tests. Multiple-group comparisons were tested by analysis of variance (ANOVA) with *post-hoc* Tukey tests. The relation between %D-Asp and age was tested by linear regression analysis. The difference in %D-Asp between N_{DEG} and control cartilage was tested by analysis of covariance (ANCOVA) using age as covariant. *p*-values < 0.05 were considered to represent statistically significant differences; absence of statistical significance is indicated by N.S. (not significant). Data are presented as individual data or as mean ± SEM.

RESULTS

Collagen residence time in N_{DEG} and control cartilage

As a measure of collagen residence time the %D-Asp was determined. In both control and N_{DEG} cartilage collagen, the %D-Asp increases with donor age ($r = 0.77$, $p < 0.001$ and $r = 0.70$, $p < 0.001$, respectively; Figure 1A), which is consistent with our previous findings.[341] Strikingly, %D-Asp levels are sig-

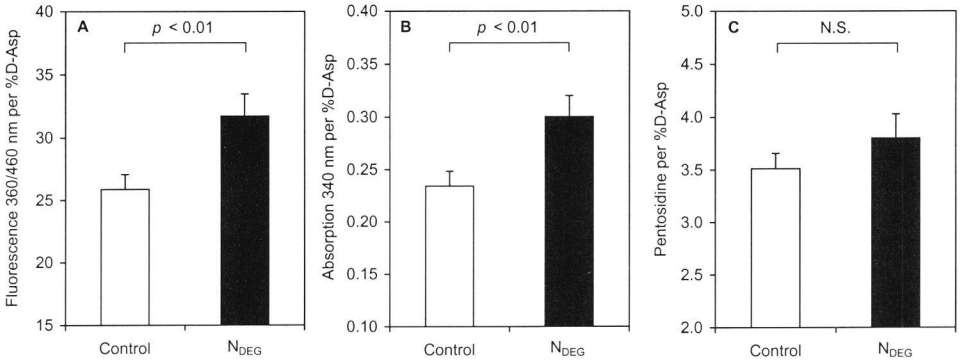

FIGURE 2 - General AGE fluorescence (flu 360/460 nm per nmol Hyp; **A**), browning (absorption 340 nm per µmol Hyp; **B**), and pentosidine (mmol/mol collagen; **C**), corrected for the collagen residence time (%D-Asp) in collagen from control cartilage and normal cartilage from knees with focal degeneration (N_{DEG}). Corrected AGE levels, *i.e.* the rate of AGE formation, are higher in collagen from N_{DEG} cartilage than in control cartilage collagen.

nificantly lower in N_{DEG} cartilage than in control cartilage ($p < 0.001$), indicating that the residence time of collagen in the macroscopically normal (N_{DEG}) cartilage from donors with focal cartilage degeneration is lower than in normal cartilage from unaffected controls, *i.e.* the rate of collagen turnover is increased.

Based on the %D-Asp results a half-life for control cartilage collagen of 88 years was calculated (95% confidence interval: 63-136 years; Figure 1B). This value is in good agreement with the previously determined value of 117 years that was based on a more extensive age range.[341] The mean half-life of collagen in N_{DEG} cartilage was significantly lower and was only 43 years (95% confidence interval: 35-54 years; $p < 0.001$).

AGE cross-linking of collagen from N_{DEG} and control cartilage

Levels of AGE cross-links were measured as fluorescence (at 360/460 nm), browning (absorption at 340 nm) and pentosidine in N_{DEG} and control cartilage collagen. Consistent with our previous data,[340] all measures of AGE cross-linking increased with increasing age of the donor (data not shown). Because collagen AGE levels are greatly influenced by the rate of collagen turnover,[341] the increased rate of collagen turnover in N_{DEG} cartilage compared to control cartilage (Figure 1) confounds the comparison of absolute AGE levels. To correct AGE contents for the difference in collagen turnover, AGE levels per %D-asp were calculated. This ratio provides us with a modified measure for the AGE content of cartilage collagen, namely the AGE level corrected for collagen turnover, *i.e.* the rate of AGE formation.

The rates of formation of AGE fluorescence and browning were substantially higher in N_{DEG} cartilage than in control cartilage: fluorescence in N_{DEG} cartilage collagen was 23% higher than in control cartilage, browning was 28% higher ($p < 0.01$ in both cases; Figure 2). Pentosidine per %D-asp was 8% higher in N_{DEG} cartilage compared to control cartilage, but this difference did not reach statistical significance.

Integrity of the collagen network in N_{DEG} and control cartilage

Because enzymatic posttranslational modifications of collagen may affect the quality of the cartilage collagen network,[34] lysyl hydroxylation and pyridinoline cross-linking were also compared between N_{DEG} and control cartilage. Both hydroxylysine (Hyl) and hydroxylysylpyridinoline (HP) levels remained constant with increasing age of the donor[25] and were identical in the two groups (both N.S.; Figure 3). The latter finding indicates that levels of enzymatic modifications do not

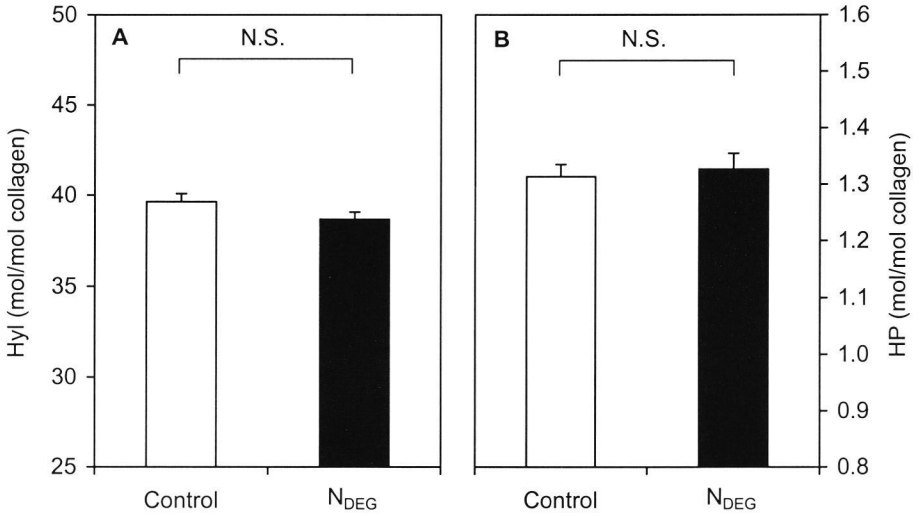

FIGURE 3 - Hydroxylysine (Hyl; **A**) and hydroxylysylpyridinoline (HP; **B**) levels in collagen from control cartilage and normal cartilage from knees with focal degeneration (N_{DEG}). Enzymatic modifications of collagen from N_{DEG} cartilage and control cartilage are similar.

confound our study into possible effects of AGEs. Furthermore, these results indicate that despite the substantially increased turnover in N_{DEG} cartilage collagen, the newly synthesized collagen has the same extent of enzymatic modifications (both Hyl and HP) as control cartilage collagen and is properly cross-linked into a network (HP).

The isolation of cartilage collagen - as performed in the present study - involves removal of the proteoglycans and other non-collagenous proteins from the tissue by sequential enzymatic treatment with chondroitinase ABC, trypsin and hyaluronidase.[289] Especially the treatment with trypsin could result in loss of collagen from the samples during the incubation. Collagen loss during the three subsequent enzyme incubations was measured in subsets of pairs of DEG and N_{DEG} cartilage (n=4 pairs; aged 62-77 years) and of control cartilage (n=7; aged 62-83 years). The collagen that was lost during the enzyme treatments was predominantly lost during the trypsin step irrespective of the type of cartilage (data not shown). Overall collagen loss was identical in N_{DEG} and control cartilage (N.S.), and, as expected, significantly higher in DEG cartilage ($p < 0.05$). The fact that from N_{DEG} and control cartilage only a small and comparable amount of collagen is lost during the enzyme treatments indicates that our comparison of collagen from these two sources is not confounded by either extensive loss or a difference in loss of collagen during sample preparation.

Actually, the data from trypsin treatment (Figure 4A) provide a similar measure for damaged collagen as can be obtained from measuring the α-chymotrypsin sensitive collagen.[29] The high collagen release from DEG cartilage upon trypsin incubation results from the presence of a substantial amount of denatured collagen in the samples.[29] The low level of trypsin-sensitive collagen in N_{DEG} and control cartilage shows that collagen triple helices are equally intact in N_{DEG} and control cartilage. As indicated in Figure 4B, the residence time of collagen in the subset of N_{DEG} and DEG cartilage samples is significantly lower than in the subset of control samples ($p < 0.01$; Figure 4B). The ages of the control and N_{DEG}/DEG cartilage donors were comparable (75.6 ± 2.9 and 69.3 ± 3.7 years, respectively; N.S.). In conclusion, these results indicate that in-

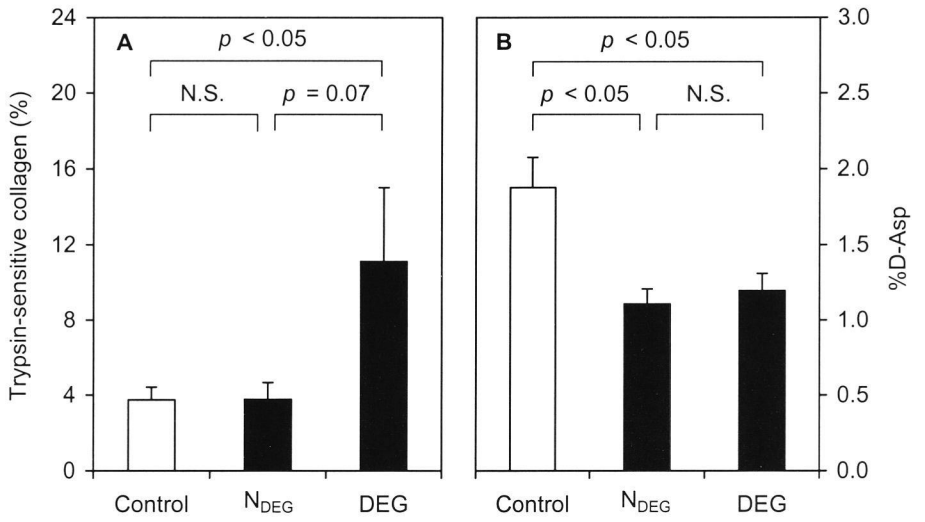

FIGURE 4 - The amount of trypsin-sensitive collagen (% of total collagen; **A**) and collagen aspartic acid racemization (%D-Asp; **B**) in a subset of control cartilage (n=7) and in pairs of normal (N_{DEG}) and degenerated (DEG) cartilage from knees with focal degeneration (n=4). The amount of trypsin-sensitive collagen (indicative of the amount of damaged collagen) is increased only in DEG cartilage, while collagen turnover is increased in both N_{DEG} and DEG cartilage.

creased collagen turnover in N_{DEG} cartilage (Figure 4B) resulted in considerably "younger" collagen that is intact and normally integrated in the network (Figure 4A).

DISCUSSION

We have previously postulated that the age-related increase in AGE cross-linking of articular cartilage collagen plays a role in the increase in OA incidence with age.[25,83,340] In the present cross-sectional study, AGEs were compared between visually intact cartilage from individuals with focal cartilage degeneration (N_{DEG}) and cartilage from age-matched individuals without any sign of cartilage degeneration (control).

Although we limited our comparison to macroscopically (and histologically) normal cartilage, the residence time of collagen in N_{DEG} cartilage was substantially lower than in control cartilage, *i.e.* the rate of turnover of collagen is increased in N_{DEG} cartilage. N_{DEG} cartilage was obtained from knees in which only mild to moderate focal cartilage degeneration was observed (Mankin grade of the lesions was only 5-6 compared to 9-10 in clinical OA).[200,334] At first sight, such focal lesions are not expected to affect the metabolism in normal cartilage remote from the lesion. Indeed, we have previously shown that N_{DEG} cartilage has a Mankin grade (0-1) and proteoglycan metabolism comparable to control cartilage.[180] In fact, even the visually intact cartilage from knees displaying clinical OA has been shown to be comparable to cartilage from control joints with respect to swelling, proteoglycan content, proteoglycan metabolism, and collagenase activity, indicating that degenerative changes are focal in origin.[58,113] In contrast, others have reported decreased stiffness and proteoglycan content and upregulated type II collagen expression in macroscopically normal cartilage remote from localized areas of degeneration.[7,159,161] Our present data indicate increased collagen turnover in N_{DEG} cartilage and consequently that the visually intact cartilage in joints with focal degenerative changes is in that respect not "normal". Nevertheless, despite its increased turnover, the integrity of the collagen network in N_{DEG} cartilage can still be

considered normal. This suggests, contradicting current belief,[8,119] that chondrocytes in adult human articular cartilage are able to remodel the collagen network extensively and maintain a functional collagen network.
As collagen turnover is a key determinant of AGE levels,[341] the residence time of the collagen needs to be taken into account when comparing AGE contents of collagens with different turnover rates. Consequently, we calculated the AGE level corrected for residence time, *i.e.* the AGE formation rate, to provide a valid measure for comparison of AGEs between N_{DEG} and control cartilage collagen. AGE formation rates were significantly higher in N_{DEG} cartilage than in control cartilage, indicating that a high rate of AGE formation in cartilage collagen is related to the occurrence of focal cartilage degeneration.

These results, combined with the observed increase in collagen turnover in N_{DEG} cartilage, may provide new insight into the role of cartilage collagen network properties in the development of OA. The explanation of our results strongly depends on whether the observed increase in collagen turnover in N_{DEG} cartilage is regarded a primary or a secondary event in the development of OA. Inasmuch as our cross-sectional study is inconclusive on this, we discuss two possible explanations of our results in the light of OA development.

On the one hand, it could be that collagen turnover was increased prior to the development of lesions and therefore in itself presents a risk factor for cartilage degeneration at old age. This would imply that the high rate of AGE formation and increased collagen turnover in N_{DEG} cartilage independently contribute to the development of OA. At present, we have no indication of the mechanism through which increased cartilage collagen turnover may lead to OA, especially since the normal integrity of the collagen network is maintained. Nevertheless, these results provide interesting new information on cartilage properties related to the development of OA that merit further attention.

On the other hand, assuming that the increase in collagen turnover in N_{DEG} cartilage occurs secondary to the disease process, the presence of both a high AGE formation rate and increased collagen turnover in N_{DEG} cartilage may be explained as follows. The high rate of AGE formation leads to substantial AGE cross-linking of cartilage collagen which results in increased stiffness of the collagen network[25] up to the level where the collagen network becomes brittle, *i.e.* more sensitive to damage.[68] The increased sensitivity to damage eventually results in the development of cartilage degeneration. As a consequence of the degenerative process, collagen turnover increases in the entire joint as is substantiated by the increased collagen turnover in N_{DEG} cartilage. Since levels of AGEs and %D-Asp are equally reduced by increased turnover,[341] the AGE level corrected for turnover, *i.e.* the rate of AGE formation, reflects the extent of AGE cross-linking of the collagen network in N_{DEG} cartilage prior to the start of the degenerative process. In this line of reasoning the high rate of AGE formation in cartilage collagen is the major risk factor for OA identified in the present study. Since the rate of AGE formation is largely determined by sugar concentration,[83] our data are consistent with higher blood glucose levels in patients with symptomatic OA compared to healthy controls.[71] It would be interesting to learn whether the increased rate of AGE formation in cartilage from OA-affected individuals reflects a generalized process which also affects other tissues such as skin. If that were the case, measures of AGEs in skin or blood may provide new markers for OA risk assessment.[292,293]

In conclusion, the present results show that a high rate of AGE formation in cartilage collagen is related to the occurrence of focal cartilage degeneration. This supports the hypothesis that collagen AGE cross-links predispose to the development of OA. Thus, the age-related accumulation of AGE cross-links presents a putative molecular mechanism whereby age contributes to the risk to develop OA. Furthermore, the novel

observation of increased collagen turnover in macroscopically normal cartilage of OA-affected individuals suggests that, probably as a result of the initial cartilage damage, adult human articular cartilage is able to remodel its collagen network while maintaining normal integrity.

ACKNOWLEDGEMENTS

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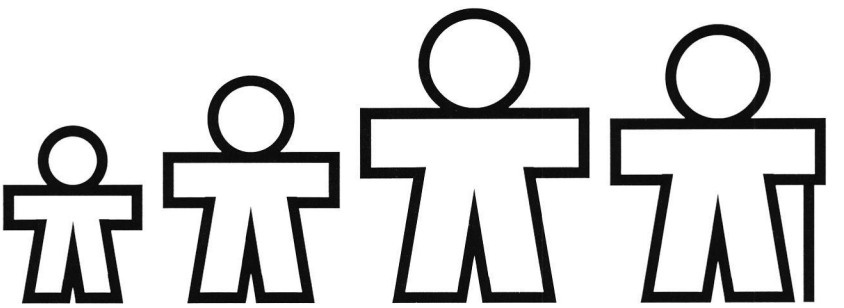

Chapter 7

Putative Role of Lysyl Hydroxylation and Pyridinoline Cross-Linking During Adolescence in the Occurrence of Osteoarthritis at Old Age

Ruud A. Bank,[a,b] Nicole Verzijl,[a] Floris P.J.G. Lafeber[c] and Johan M. TeKoppele[a]

Objective: *The collagen network in human articular cartilage experiences a large number of stress cycles during life as it shows hardly any turnover after adolescence. We hypothesized that, to withstand fatigue failure, the physical condition of the collagen network laid down at adolescence is of crucial importance for the age of onset of osteoarthritis (OA).*

Methods: *We have compared the lysyl hydroxylation level and pyridinoline cross-link level of the collagen network of degenerated (DEG) cartilage of the femoral knee condyle (representing a preclinical early stage of OA) with that of normal cartilage from the contralateral knee. The biological age of the collagen network was determined by means of pentosidine levels. For each donor, collagen modifications of normal cartilage were compared with DEG cartilage that showed no significant remodeling of the collagen network (as evidenced by identical pentosidine levels).*

Results: *DEG cartilage contained significantly more hydroxylysine residues per collagen molecule in comparison to healthy cartilage from the same donor, both in the upper and lower half (the region near the articular surface and adjacent to bone, respectively). In addition, a significantly higher level of pyridinoline cross-linking was observed in the upper half of DEG cartilage. Considering the biological age of the collagen network, the changes observed in DEG cartilage must have been present several decades before cartilage became degenerated.*

Conclusions: *The data suggest that high levels of lysyl hydroxylation and pyridinoline cross-linking result in a collagen network that fails mechanically in long term loading. Areas containing collagen with low hydroxylysine and pyridinoline levels are less prone to degeneration. As such, this study indicates that posttranslational modifications of collagen molecules synthesized during adolescence are causally involved in the pathogenesis of OA.*

SUBMITTED FOR PUBLICATION

Introduction

The extracellular matrix of cartilage is essentially a fiber-reinforced gel, containing highly negatively charged proteoglycans entangled in a network of collagen fibrils. Proteoglycans have, because of their intrinsic property to attract water, the tendency to swell. The tensile stiffness of the collagen network counteracts this swelling. It is this delicate balance which endows cartilage with its load-bearing properties.[62,207] As a consequence of the swelling properties of proteoglycans, collagen is under constant tension, even when cartilage is unloaded. When loaded, e.g. during standing or walking, joint cartilage undergoes high compressive forces, ranging from 40 to 200 atmospheres.[2] Loading is accompanied by a displacement of proteoglycans; this bulk movement of the proteoglycan-water gel is resisted by the collagen network.[224] By doing so, the collagen network must experience increased tensile stresses. Especially under cyclic loading, such as in walking, the collagen network is continuously exposed to stress cycles.

The collagen network of articular cartilage consists of several collagen types, namely collagen type II, IX and XI.[99] One of the earliest changes seen in osteoarthritis (OA) is an increase in water content in articular cartilage.[202] This is due to a damaged collagen network, resulting in a loss of tensile stiffness.[31,35,202] Several decades ago, Freeman suggested that the primary event in the pathogenesis of idiopathic (primary) OA is a collagen fatigue failure.[112] Fatigue refers

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to the failure of a material because of repetitive stressing at a level below the ultimate strength of the material. The fatigue theory has been investigated in detail by Weightman.[350-352] An important feature of the collagen network in adult human articular cartilage is that it hardly shows turnover.[25,341] Thus, the collagen network experiences a large number of stress cycles during life. We hypothesize therefore that, to withstand fatigue, the physical condition of the collagen network laid down at adolescence is of crucial importance towards the age of onset of OA.

An implication of the above hypothesis is that a qualitatively inferior collagen network results in early-onset OA. Indeed, mutations have been found in collagen type II, IX and XI in hereditary osteochondrodysplasias (such as spondyloepiphyseal dysplasia, Marshall and Stickler syndrome) that are associated with severe and early-onset OA.[120,177,250,310,311,359] More interestingly, two recurrent point mutations ($Arg_{75}\rightarrow Cys$ and $Arg_{519}\rightarrow Cys$) and a reduced expression of collagen type II have been found in patients with early-onset, primary, generalized OA.[9,47,48,98,140,195,271,275,276,358-360] In these cases, the development of OA does not involve anatomic abnormalities, and is primarily due to an impaired collagen network. As a matter of fact, the role of collagen mutations in the development of OA has been confirmed in transgenic mice studies,[100,125,137,239,279] once more highlighting the important role of a reduction of collagen fibril quality in the pathogenesis of early-onset OA.

It has been found that the $Arg_{519}\rightarrow Cys$ mutation in the α1(II) chain results in an increased level of triple helical lysyl hydroxylation of collagen type II.[98] A higher lysyl hydroxylation level of collagen has been found in a variety of pathological conditions, such as osteogenesis imperfecta, osteoporosis, and osteochondrodysplasias.[32,175,235] A relationship has been established between overmodification and phenotypic severity of the disease: the higher the level of lysyl hydroxylation, the more severe the phenotype.[235] A higher lysyl hydroxylation level is not always the result of collagen mutations, but can also be due to e.g. increased levels of lysyl hydroxylase.[55,186] We hypothesize that an increase in lysyl hydroxylation of the triple helix results in subtle changes of the collagen network, giving rise to a qualitatively inferior network. If so, lysyl hydroxylation might play a role in the age of onset of OA: a collagen network with high hydroxylysine levels might be more prone to fatigue.

Macroscopically fibrillated cartilage is frequently observed in the knee of donors without a clinical history of OA or other joint disorders.[101] This degenerated (DEG) cartilage, containing increased levels of damaged collagen molecules, can be considered an early, preclinical phase of OA.[334] In this study, we have compared within each donor the posttranslational modifications (lysyl hydroxylation and pyridinoline cross-linking) of collagen as well as the proteoglycan content of normal and DEG knee cartilage. To ensure that the collagen network of normal and DEG cartilage of a donor was of the same biological age, pentosidine levels were measured. Pentosidine is a cross-link resulting from non-enzymatic glycation. It accumulates linearly with age in cartilage after the age of 20 years, which is consistent with the long half-life (around 100-200 years) of collagen in adult cartilage.[25,341] Pentosidine serves as a suitable biomarker to estimate the age and the remodeling of the collagen network.[25,33,340,341] Knowing the biological age of the collagen network between normal and DEG cartilage of each donor is of particular importance: if the collagen network in DEG cartilage is of a younger age than that of normal cartilage, it is difficult to discriminate whether changes in lysyl hydroxylation and pyridinoline cross-linking in DEG cartilage are of a secondary nature (*i.e.* changes due to new collagen synthesis) or a primary event involved in the degeneration. When the collagen network is of the same biological age, the extremely slow turnover of the collagen network after adolescence implies that differences in posttranslational modifications between normal and DEG

collagen precede OA pathology by several decades. Thus, in the latter case, differences in posttranslational modifications may have played a causative role in the predisposition of the collagen network to fatigue.

EXPERIMENTAL PROCEDURES

Cartilage

Normal and degenerated (DEG) cartilage were obtained *post mortem* at autopsy from weight bearing areas of human femoral knee condyles within 24 h after death of the donor. The age of the donors (n = 6) was 49, 60, 72, 83, 86 and 92 years. None of the donors had a clinical history of joint disorders. The cartilage was cut with a scalpel blade, excluding the underlying bone. DEG cartilage, designated by macroscopic focal fibrillation of the articular surface, was obtained from the lateral or medial condyle and represents a preclinical stage of OA.[334] Normal cartilage, designated by a glossy, white, completely smooth surface and a healthy appearance, was obtained from a comparable position of the contralateral knee from the same donor. All cartilage cubes were divided in an upper half (the region near the articular surface) and a lower half (the region adjacent to the bone). From each donor (n = 6) three normal and three degenerated pieces of cartilage were obtained. This resulted in 36 cartilage specimens from the upper part and 36 from the lower part (18 normal and 18 DEG specimens).

Lysyl hydroxylation and cross-linking of collagen

Normal and degenerated cartilage pieces (routinely 1-2 mg dry weight) were hydrolyzed (108°C, 20-24 h) with 6 M HCl in teflon-sealed glass tubes. The hydrolyzed samples were dried and redissolved in 200 µl water containing 10 µM pyridoxine (internal standard for the cross-links) and 2.4 mM homoarginine (internal standard for amino acids) (Sigma, St. Louis, MO). Samples were diluted 5-fold with 0.5% (v/v) heptafluorobutyric acid (Fluka, Buchs, Switzerland) in 10% (v/v) acetonitrile for cross-link analysis; aliquots of the 5-fold diluted sample were diluted 50-fold with 0.1 M sodium borate buffer pH 8.0 for amino acid analysis. Derivatization of the amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography of amino acids (hydroxyproline, Hyp, hydroxylysine, Hyl, and proline, Pro) and cross-links (hydroxylysylpyridinoline, HP, lysylpyridinoline, LP, and pentosidine) were performed on a Micropak ODS-80TM column (150 x 4.6 mm; Varian, Sunnyvale, U.S.A.) as described previously.[27,28] The quantities of cross-links as well as Hyl were expressed as the number of residues per collagen molecule, assuming 300 Hyp residues per triple helix. As acid hydrolysis converts glucosylgalactosylhydroxylysine and galactosylhydroxylysine into hydroxylysine in a stoichiometric way, the data reflect total hydroxylysine levels. Although it is known that pentosidine is present in non-collagenous proteins as well, no attempt was made to correct for this release, as pentosidine in cartilage hydrolysates predominantly originates from the collagen network.[Chapter 4] No attempt was made to deplete the proteoglycans from the cartilage, because we were interested in the amount of proteoglycans in the cartilage as well. The relative amount of proteoglycans was estimated by the ratio Hyp/Pro as we reasoned that the lower the Hyp/Pro ratio the more non-collagenous proteins (mainly proteoglycans) are present in the tissue. The relationship between proteoglycan content and Hyp/Pro ratio was tested by comparing the fixed charge density (FCD) and the Hyp/Pro in a separate set of samples.

Determination of fixed charge density (FCD)

The FCD is defined as the concentration of fixed negatively charged groups (chondroitin sulfate and keratan sulfate) in the tissue (expressed as mmol/g tissue). The relationship between FCD and Hyp/Pro ratio was determined in a series of full-depth cartilage plugs derived from normal human femoral heads obtained *post mortem* at autopsy. The

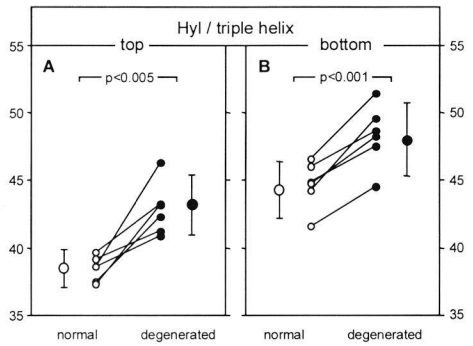

FIGURE 1 - Variation in hydroxylysine (Hyl) level of collagen (expressed as number of residues per triple helix) in normal and degenerated cartilage. Cartilage was divided in an upper half ('top': the region near the articular surface) and a lower half ('bottom': the region adjacent to the bone). Lines connect normal and degenerated cartilage from one donor.

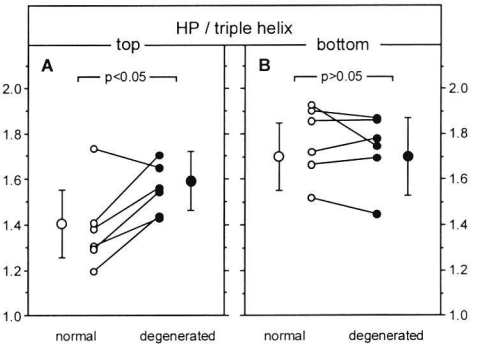

FIGURE 2 - Variation in hydroxylysylpyridinoline (HP) level of collagen (expressed as number of cross-links per triple helix) in normal and degenerated cartilage. Cartilage was divided in an upper half ('top': the region near the articular surface) and a lower half ('bottom': the region adjacent to the bone). Lines connect normal and degenerated cartilage from one donor.

FCD was determined as described by Maroudas and co-workers.[31] After washing out the radio-active tracer, the specimens were freeze-dried to constant weight; subsequently, amino acid analysis was performed as described above.

Data presentation

From each donor only samples with comparable (variation ± 10%) pentosidine levels were used to compare the posttranslational modifications of the collagen network and the Hyp/Pro ratio between DEG and normal cartilage. DEG specimens that could not be matched with normal specimens were omitted: they all showed minimally 10% lower pentosidine levels, indicating the presence of newly synthesized collagen. In the figures, each point is the mean of the replicates derived from one donor; thus, each point represents the mean of 3 replicates unless samples had to be excluded due to low pentosidine levels. Paired two-sided *t*-tests were applied to test statistical significance between DEG and normal cartilage.

RESULTS

Chondrocytes in OA cartilage exhibit increased synthesis rates of collagen.[242] The collagen network in OA cartilage may therefore contain significant amounts of newly synthesized collagen. In such event, comparing the posttranslational modifications of collagen of normal and OA cartilage is severely hampered: it is impossible to discriminate whether changes in OA are of a secondary nature (due to newly synthesized collagen in an attempt to repair the cartilage defect) or a primary event. As we wanted to compare collagen modifications (lysyl hydroxylation and pyridinoline cross-linking) in cartilage that showed no significant remodeling of the collagen network, we investigated normal and degenerated femoral condyle cartilage obtained from donors without a clinical history for joint diseases. This degenerated cartilage represents a preclinical stage of OA[334] and was expected to contain little newly synthesized collagen. Indeed, in all six donors DEG cartilage was found that contained negligible amounts of newly synthesized collagen (as evidenced by pentosidine levels). Consequently, the collagen network in these cartilage specimens is of the same biological age as that of normal cartilage, *i.e.* it was laid down at age < 20 years.

A paired Student's *t*-test revealed significantly higher levels of Hyl in the upper and lower part of DEG cartilage compared to

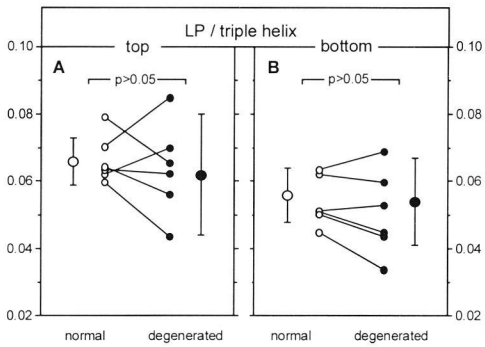

FIGURE 3 - Variation in lysylpyridinoline (LP) level of collagen (expressed as number of cross-links per triple helix) in normal and degenerated cartilage. Cartilage was divided in an upper half ('top': the region near the articular surface) and a lower half ('bottom': the region adjacent to the bone). Lines connect normal and degenerated cartilage from one donor.

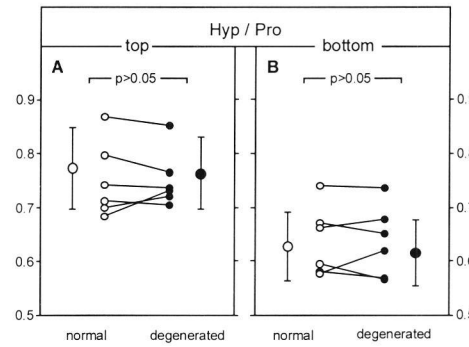

FIGURE 4 - Variation in the Hyp/Pro ratio (indicative for the amount of non-collagenous proteins compared to collagen) in normal and degenerated cartilage. Cartilage was divided in an upper half ('top': the region near the articular surface) and a lower half ('bottom': the region adjacent to the bone). Lines connect normal and degenerated cartilage from one donor.

normal cartilage ($p < 0.005$ and < 0.001, respectively; Figure 1). The Hyl levels in the upper and lower part of DEG cartilage are 12 ± 7% and 8 ± 3% (mean ± S.D.) higher than in normal cartilage, respectively. Furthermore, a significantly higher HP level is found in the upper part of DEG cartilage compared to normal cartilage ($p < 0.05$; Figure 2). The HP level in the upper part of DEG cartilage is 14 ± 11% higher than in control tissue. In contrast, the HP level of the collagen molecules in DEG cartilage from the lower part is the same as in normal cartilage (Figure 2). There were no significant differences between DEG and normal cartilage with respect to the amount of LP, neither in the upper, nor in the lower part (Figure 3).

As there are no age-related differences in the enzymatic modifications of collagen (lysyl hydroxylation and pyridinoline cross-linking),[25] the values of the six donors can be pooled despite the considerable age range (49-92 years). The mean ± S.D. of the amount of Hyl, HP, and LP per collagen molecule for the normal upper part (degenerated upper part) is 38.5 ± 1.4 (43.2 ± 2.2), 1.41 ± 0.15 (1.60 ± 0.13) and 0.066 ± 0.007 (0.062 ± 0.018), respectively. The mean ± S.D. of the amount of Hyl, HP, and LP per collagen molecule for the normal lower part (degenerated lower part) is 44.3 ± 2.1 (48.0 ± 2.7), 1.70 ± 0.15 (1.70 ± 0.17) and 0.056 ± 0.008 (0.054 ± 0.013), respectively. Consistent with our previous findings,[25] the amount of Hyl and HP in normal cartilage increased from the articular surface to the bone, whereas for LP the reverse is seen. The same zonal variation was found in DEG cartilage.

We reasoned that the ratio Hyp/Pro is indicative for the amount of non-collagenous proteins (such as proteoglycans) compared to collagen. Increased proteoglycan levels should result in increased Pro levels compared to Hyp (giving rise to lower Hyp/Pro ratios). This was tested in full-depth cartilage derived from normal femoral heads. Indeed a strong correlation ($r = 0.961$; $p < 0.001$) was found between the FCD (mmol/g dry tissue) and the Hyp/Pro ratio (Figure 5). The Hyp/Pro ratio in the upper part of normal (degenerated) cartilage is 0.772 ± 0.077 (0.763 ± 0.067); in the lower part of normal (degenerated) cartilage it is 0.627 ± 0.064 (0.615 ± 0.062) (Figure 4). This is consistent with the observation, that there are more proteoglycans in the deep zone than in the surface region.[122] There was no significant difference between the Hyp/Pro

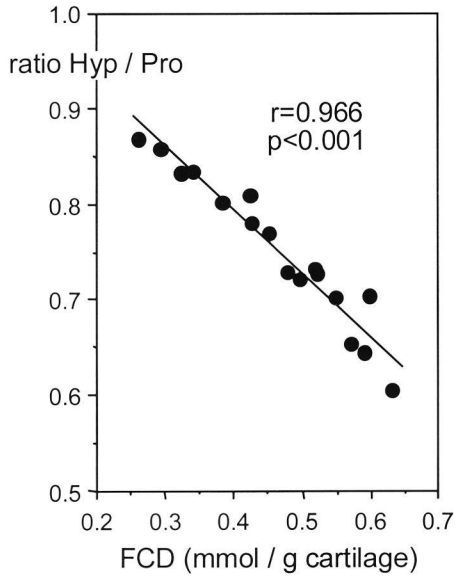

FIGURE 5 - Relation between the fixed charge density (FCD) and Hyp/Pro ratio of cartilage. The good correlation shows that the Hyp/Pro ratio can be used as a measure of the proteoglycan content of cartilage.

ratio of normal and degenerated cartilage (Figure 4), indicating that in normal and DEG cartilage the amount of proteoglycans compared to the amount of collagen is the same.

DISCUSSION

Because of their high osmotic pressure proteoglycans are able to swell almost indefinitely. The swelling tendency of proteoglycans is limited by, and hence induces tensile stresses in, the collagen network.[35,207,224] The higher the proteoglycan concentration, the higher the tensile stresses in the collagen network will be. It is conceivable that higher tensile stresses make collagen more prone to fatigue. In our samples, the proteoglycan content between DEG and normal cartilage were comparable. It thus seems unlikely that this phenomenon plays a role in the impairment of the collagen network of the studied DEG cartilage samples. It is therefore reasonable to assume that the defect is located in the collagen network itself, the major structural component of cartilage.

In adult human articular cartilage, the half-life of the collagen network is in the order of 100-200 years.[341] Over several decades, pentosidine accumulates in articular collagen.[25,341] As a result, *de novo* synthesis of collagen (as occurs in OA cartilage), will result in decreased amounts of pentosidine per collagen molecule.[33,Chapter 4] Specimens were selected showing no differences in pentosidine levels between DEG and normal cartilage obtained from the same donor. In these specimens, comparison of DEG and normal cartilage is not confounded by *de novo* synthesis of collagen (*i.e.* the collagen network in DEG cartilage reflects the situation before the onset of fibrillation). Significantly more hydroxylysine residues and HP cross-links were present per collagen molecule in the upper part of DEG cartilage than in healthy cartilage. In addition, significantly more hydroxylysine residues per collagen molecule were found in the lower part of DEG cartilage. Since these differences precede cartilage fibrillation, lysyl hydroxylation (and likely pyridinoline cross-linking as well) may be causally involved in the mechanically-induced cartilage degeneration due to collagen fatigue. An increased level of damaged collagen molecules has been found in osteoarthritic and degenerated cartilage, the highest concentrations being localized in the upper part.[29,141] Indeed, the largest differences in enzymatic modifications between degenerated and normal cartilage are observed in the upper part. This suggests a direct causal relation between collagen modifications and mechanical fatigue failure seen in preclinical OA cartilage.

As noted before, Hyl and HP increase with depth from the surface in the collagen in articular cartilage.[25] By sampling the top half and the bottom half of the tissue, it could be argued that the differences in Figures 1A-B and 2A are explained by loss of the top half of the degenerated tissue over time. In that case, the deeper tissue is now sampled as the "surface" of degenerated cartilage and compared to the actual surface of healthy cartilage. However, if this is the case, one would expect to see differences

between degenerated and normal cartilage in LP and Hyp/Pro ratios as well, as gradients have been found for LP[25] and proteoglycan concentration.[122] Since this is not the case (Figures 3-4), it is unlikely that in our samples erosion of the original surface of degenerated cartilage took place.

Hydroxylation of triple helical lysyl residues (including the attachment of the glycosides glucose and galactose on hydroxylysine) seems to play a crucial role in the formation of a properly functioning collagen network. A decrease in Hyl levels results in severe alterations of the ECM organization, e.g. in Ehlers-Danlos type VI syndrome.[151] A relationship between lysyl overhydroxylation and phenotypic severity has been found in osteogenesis imperfecta and osteochondrodysplasias.[235] In addition, a close relationship has been found between the degree of collagen overmodification and the severity of osteopenia.[36] Eyre *et al.*[98] noted a lysyl overhydroxylation in early-onset OA cartilage exhibiting the $Arg_{519} \rightarrow Cys$ mutation. Also Kashin-Beck disease, an endemic, chronic and degenerative osteoarticular disorder found in China, is instructive of the role of collagen modification in OA. This is an acquired rather than an inherited disease: selenium deficiency in the diet and a high content of fulvic acid are the main causative factors of the disease.[363,364] Cartilage of Kashin-Beck patients showed increased levels of hydroxylysine.[364] An increase in lysyl hydroxylation was also seen in selenium-deficient mice and fulvic acid-supplemented mice.[363]

A failure of the collagen network is the principal postulate in many mechanical hypotheses of OA. We have recently argued that accumulation of non-enzymatic glycation products (such as pentosidine) in the collagen network contribute to the age-related decrease in the resistance of cartilage to fatigue.[25,340] Superimposed on these age-related non-enzymatic modifications, we now provide evidence that the "starting quality" of the collagen network (as laid down in the second decade of life) determines the susceptibility of cartilage to failure as well. The increased level of the cross-link HP in the upper part of degenerated cartilage may lead to changes in the physical properties of cartilage, making the collagen in the top layer more prone to mechanical breakdown following normal (repetitive) loading. In addition, lysyl overhydroxylation seems to alter the fibrillar structure of the collagen network, resulting in an impairment of the functional integrity of the extracellular matrix. Altogether, changes in modifications of the articular cartilage collagen network (either enzymatic or non-enzymatic) seem to reduce its quality: the altered fibrils may be less able in the long term to withstand the mechanical stresses that articular cartilage endures throughout human adult life. Interventions aimed at altering the fatigue properties of the cartilage collagen network are expected to delay the age of onset of osteoarthritis, thus enhancing the quality of life.

ACKNOWLEDGEMENTS

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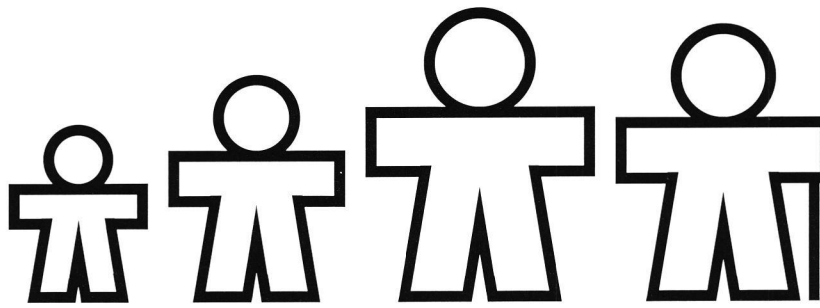


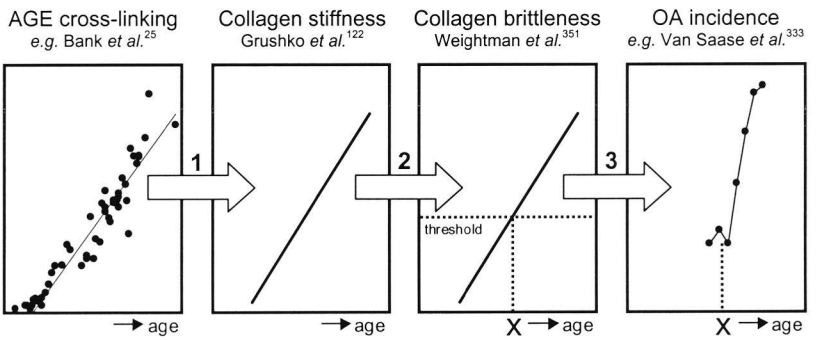
Chapter 8

SUMMARY AND DISCUSSION

Osteoarthritis (OA), or cartilage degeneration, is a chronic disabling disease for which age is the single greatest risk factor.[78,105] Although the mechanism through which aging is involved is largely unknown, age-related changes in articular cartilage are expected to play an important role in increasing the susceptibility for cartilage degeneration.[25,54,158] A prominent age-related change in human articular cartilage is the increase in the level of pentosidine,[25,331] an advanced glycation endproduct (AGE) that forms cross-links between proteins. In addition, there is an age-related increase in the stiffness of the articular cartilage collagen network[35,122] and collagen damage is the first sign of cartilage degeneration.[202] Moreover, the resistance of the cartilage collagen network to fail mechanical failure decreases with age.[157,350] This led to the following hypothesis: the age-related increase in AGE cross-linking in cartilage collagen results in increased stiffness of the collagen network up to the level where the collagen network becomes brittle, i.e. *more sensitive to damage, which results in an increased risk to develop OA (Figure 1).*

To test this hypothesis, we have investigated the biochemistry of AGE accumulation in human adult articular cartilage and the effect of AGE accumulation on several cartilage properties that are important for maintenance of the integrity of the tissue, such as collagen network stiffness, chondrocyte metabolism, and proteoglycan and collagen turnover. Furthermore, the effect of AGE cross-linking on the sensitivity to develop OA was studied in a canine in vivo *model of OA and in a cross-sectional population study. The results of these studies are in part described in the thesis of Jeroen DeGroot: "Advanced glycation endproducts in the development of osteoarthritis: cartilage synthesis and degradation".[82] The focus of the present thesis was on the biochemistry of AGE accumulation in human adult articular cartilage, including the role of protein turnover, and the effect of AGE cross-linking on the mechanical properties of the cartilage collagen network. Finally, the hypothesis that AGE accumulation contributes to the age-related increase in OA incidence was tested in a cross-sectional population study investigating the association of accumulation of AGEs with development of OA. The eventual result of age-related changes in cartilage collagen also depends on the "starting quality" of the collagen network at adolescence, after which the turnover of collagen decreases, and consequently the ability to adapt to changes in functional requirements. Therefore, enzymatic modifications of the cartilage collagen network as it is laid down at adolescence were also related to the development of OA at old age. The results of these studies are further discussed in this chapter.*

FIGURE 1 - Schematic representation of the mechanism through which the age-related increase in AGE content in human adult articular cartilage may contribute to the increased risk to develop osteoarthrtitis (OA) with increasing age. From left to right, the relation between cartilage AGE cross-linking, collagen stiffness, collagen brittleness and OA incidence (y-axis) are plotted versus donor age (x-axis). 1. The age-related increase in AGE cross-linking results in increased stiffness of the cartilage collagen network. 2. The increased stiffness of the cartilage collagen network results in an increase in brittleness, i.e. sensitivity to damage. After a certain level of brittleness is reached (threshold), the collagen becomes increasingly damaged (age "X"). 3. From age "X" onwards the risk to develop OA increases, which is illustrated by the age-related increase in OA incidence.



AGE-RELATED ACCUMULATION OF MAILLARD REACTION PRODUCTS IN ARTICULAR CARTILAGE COLLAGEN

In comparison to other collagen-rich tissues such as skin, cartilage contains relatively large amounts of pentosidine.[295] Since pentosidine can be considered a marker of the overall process of glycation and AGE formation,[92,295] this suggests the presence of high levels of Maillard reaction products in cartilage. Our analysis of several AGE measures in human articular cartilage collagen and the comparison of cartilage AGE levels to skin collagen and lens proteins - both also typically long-lived proteins that accumulate high amounts of AGEs - revealed that indeed cartilage collagen contains high AGE levels (**chapter 2**). This was most strikingly shown by the age-related decrease in the content of unmodified (hydroxy-)lysine and arginine residues in articular cartilage collagen (up to 10%), which is the result of an increase in modification of these glycation-sensitive amino acids. No age-related increase in amino acid modification has been observed in skin collagen or lens proteins,[86,221] indicating that overall levels of Maillard reaction products in these tissues are lower than in cartilage. Although modification of (hydroxy-)lysine residues can also be the result of enzymatic collagen cross-linking, this age-related increase in amino acid modification in cartilage collagen most likely results from glycation or oxidation processes.[25,115,223]

THE EFFECT OF PROTEIN TURNOVER ON THE ACCUMULATION OF AGEs

The fact that AGEs accumulate mainly in long-lived proteins[25,90,91,295] suggests that a slow protein turnover plays an important role in the accumulation of AGEs. Although this has often been suggested,[25,59,227,295,332] AGE levels have never been studied in relation to the rate of protein turnover. Using racemization of aspartic acid (%D-Asp) as a measure of protein residence time we were able to show that the 3-fold higher AGE levels in cartilage collagen compared to skin collagen are paralleled by 3-fold higher %D-Asp levels (**chapter 3**). The use of these %D-Asp levels to calculate the turnover rates of both collagens[208] resulted in estimated half-lives of 117 and 14 years for cartilage and skin collagen, respectively. After correction for the difference in collagen turnover, AGE levels in cartilage and skin collagen were identical, indicating that protein turnover is a key determinant in AGE accumulation.

Inasmuch as damage to the collagen network is the first sign of cartilage degeneration,[202] we have focussed our research on the collagen network in articular cartilage. Although the majority of pentosidine (80-85%) in adult human cartilage is present in collagen, pentosidine also accumulates with age in cartilage aggrecan (**chapter 4**). The fact that the pentosidine concentration in aggrecan is 3-fold lower than that in collagen, while the rate of formation of pentosidine in these two major cartilage proteins is comparable, is consistent with the faster turnover of aggrecan than of collagen.[204,208] In this chapter it was also shown that pentosidine levels can be used as a quantitative measure of protein turnover in aggrecan subfractions containing proteins that had resided in the cartilage for different lengths of time. In aggrecan isolated from OA cartilage both pentosidine and %D-Asp levels were lower than in aggrecan from normal cartilage, which is consistent with the occurrence of increased aggrecan turnover during the OA disease process.[178,182,200] In conclusion, we showed that pentosidine accumulates with age in aggrecan and that pentosidine levels can adequately be used as a quantitative measure of turnover of long-lived proteins, both during normal aging and during disease.

EFFECT OF AGE CROSS-LINKING ON THE MECHANICAL PROPERTIES OF THE COLLAGEN NETWORK IN ARTICULAR CARTILAGE

Our hypothesis that accumulation of AGEs in cartilage collagen contributes to the risk for development of OA involves a role for AGE cross-linking in increasing the stiffness

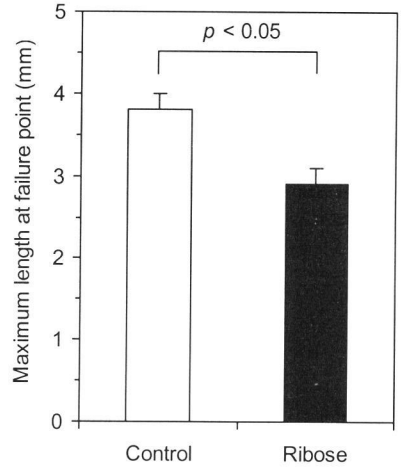


FIGURE 2 - Effect of ribose treatment on the tensile behavior of cartilage (reproduced from Chen *et al.*[68]). The failure point of a cartilage strip in tension was reached most quickly in the ribose-treated group, which indicates increased brittleness of the collagen network. Data are mean ± SEM.

(and subsequent brittleness) of cartilage collagen. To investigate the effect of cartilage collagen AGE cross-linking on the stiffness of the collagen network a study was performed in which both biochemical (AGEs) and biomechanical properties were measured specifically for collagen. Upon *in vitro* glycation of cartilage with threose, the stiffness of the collagen network increased substantially (**chapter 5**). The increase in stiffness was highly correlated to collagen AGE levels and could be prevented by specific inhibitors of glycation (lysine and arginine). In a preliminary experiment, we were also able to show that increasing the cartilage collagen AGE cross-linking by *in vitro* incubation with ribose resulted in an increase in brittleness of the collagen network.[68] This was tested by measuring tensile properties of cartilage which are considered to be primarily determined by the collagen network.[159] To test these tensile properties, a strip of cartilage is elongated until it breaks (failure point). The length of a cartilage strip at failure point was significantly decreased after ribose treatment (Figure 2), indicating that the collagen network had become more brittle.[68] Thus, based on these *in vitro* results, AGE cross-linking provides a mechanism through which the collagen network in articular cartilage becomes stiffer and more susceptible to damage with increasing age.

AGE FORMATION BY DIFFERENT GLYCATING AGENTS; COMBINED RESULTS FROM OUR *IN VIVO* AND *IN VITRO* STUDIES

The comparison of AGE levels in cartilage collagen to levels in skin collagen and lens protein revealed that different carbohydrates are responsible for glycation and AGE formation in these tissues (**chapter 2**). Based on the high levels of fructose-lysine (FL) and the high ratio of N^{ε}-(carboxymethyl)lysine (CML) to N^{ε}-(carboxyethyl)lysine (CEL) in cartilage and skin collagen, it was concluded that glucose is an important source of AGEs in these tissues. In contrast, the low FL level and the low CML/CEL ratio in lens proteins imply ascorbic acid as an important contributor to glycation and AGE formation in the lens. In addition, oxidative stress appears to be at a lower level in cartilage and skin compared to the lens based on the relatively low level of AGEs (which require oxidative processes for their formation)[41,115,116] compared to FL in cartilage and skin collagen, while the opposite was observed in lens protein.

Besides this deduction to identify which sugars are important in AGE formation in different proteins or tissues *in vivo*, insight into the diversity of AGE formation was also gained from incubations with different glycating agents *in vitro* (**chapter 5** and unpublished results). Upon incubation of cartilage with threose only 1.1% of the total amount of AGEs formed in collagen (deduced from the modification of lysine residues) could be identified as CML, CEL, or pentosidine. Incubations with ribose revealed comparable results for CML, CEL and pentosidine representing only 3.9% of all formed AGEs. Despite this similarity, threose and ribose differed largely in their CML/CEL/pentosidine 'profile', e.g. threose hardly resulted in formation of pentosidine while ribose was a very potent pentosidine source. On the

other hand, threose was a more potent source of CML and CEL than ribose. The relative amounts of CML, CEL, and pentosidine (expressed as CML:CEL:pentosidine normalized to the pentosidine content) in old human articular cartilage collagen (45:7:1) tended to resemble the profile of ribose (18:4:1) more than that of threose (72:52:1) or methylgyoxal (30:47:1). The fact that none of these *in vitro* profiles exactly matches the *in vivo* observed profile suggests that a combination of sugars is likely to contribute to the age-related increase in AGEs in human tissues.

The difference between sugars in formation of a specific AGE (*e.g.* pentosidine) *in vitro* also implies that a comparison of overall AGE levels after incubation with different sugars can not be based on levels of one of the well-characterized AGEs. Thus, when a general indication is needed of the amount of AGEs produced by a sugar, this could better be based on AGE measures such as amino acid modification and AGE fluorescence (at 360/460 nm), whereby amino acid modification measures overall glycation including AGE formation while fluorescence measures formation of cross-linking AGEs only. As expected, AGE fluorescence appeared a suitable measure to compare the effect of glycation by different sugars on stiffness of the collagen network in articular cartilage (**chapter 5**).

The results from **chapter 5**, in which threose was used as a model glycating agent, show increased stiffness of the cartilage collagen network as a result of AGE formation by threose. Inasmuch as threose is the most abundant and the most reactive degradation product of ascorbic acid,[193,237,252] a translation of these results to the *in vivo* situation may contribute to our understanding of the role of ascorbic acid in the development of OA.[212] Ascorbic acid may have a protective effect on development of OA[213,216] by its anti-oxidant properties and its beneficial effects on cartilage metabolism such as the stimulation of collagen and aggrecan synthesis.[80,172,234] In contrast, our results suggest that high levels of ascorbic acid may cause increased AGE cross-linking of the collagen network by its degradation product threose. This enhanced cross-linking results in a stiffer collagen network and likely increases the susceptibility to degeneration. Thus, ascorbic acid can potentially impact joint health in a number of ways. This issue is presently explored in a spontaneous *in vivo* OA model in guinea pigs in which the effect of high and moderate levels of ascorbic acid on the development of OA is compared to a minimal, but non-scorbutic, level of the vitamin.

AGE CROSS-LINKING OF ARTICULAR CARTILAGE COLLAGEN AS A PREDISPOSING FACTOR FOR THE DEVELOPMENT OF OA

Based on our hypothesis, cartilage collagen from individuals with OA could contain higher levels of AGE cross-links than that from individuals of comparable age without cartilage degeneration (**chapter 6**). Investigating this hypothesis has two major problems. Firstly, collagen in early stage OA cartilage has undergone higher turnover[6,154,242,258] than collagen in normal cartilage which would result in an underestimation of AGE levels in OA cartilage collagen (**chapters 3 and 4**). This artifact was circumvented by restricting our comparison to collagen from macroscopically normal cartilage from individuals with (N_{DEG}) and without (control) focal cartilage degeneration at autopsy. The second confounder - interindividual differences in cartilage collagen turnover that obscure differences in AGE levels - was overcome by taking turnover rates into account (measured as %D-Asp). Therefore, by correcting AGE levels for collagen residence time (*i.e.* AGE/%D-Asp), the AGE formation rate was determined. We showed that the AGE formation rate in collagen in N_{DEG} cartilage is higher than in control cartilage, indicating that individuals that undergo more - or more rapid - glycation are more susceptible to develop OA.

The results of this study (**chapter 6**) also led to the surprising finding of increased collagen turnover in N_{DEG} compared to con-

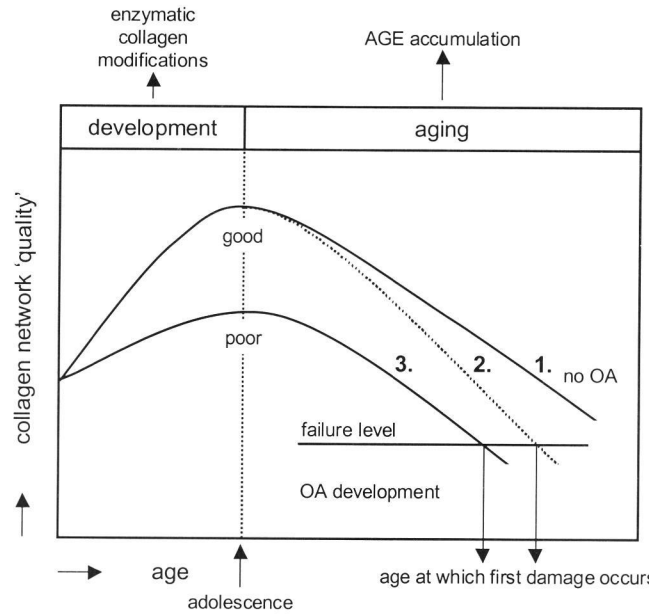


FIGURE 3 - Schematic representation of the contribution of changes in cartilage collagen during development and aging to the 'quality' of the collagen network and the risk to develop osteoarthrtitis (OA). 1. A 'normal' rate of age-related changes in collagen with a good quality at adolescence (age 20, starting quality) does not result in failure of the collagen network; no OA. **2.** Accelerated aging (*e.g.* a higher AGE formation rate) of collagen with a good starting quality results in eventual failure of the collagen network; OA develops. **3.** A 'normal' rate of deterioration of collagen network properties with age results in failure of the collagen network with a poor starting quality; OA develops.

trol cartilage, despite its normal proteoglycan metabolism and low Mankin grade (0-1; as in healthy cartilage).[180] Furthermore, as evidenced by normal Hyl and HP cross-link levels and lack of sensitivity to degradation by trypsin, the integrity of the collagen network itself was not affected by the increased turnover. Apparently, contradicting current belief,[119] chondrocytes in adult human articular cartilage are able to remodel the collagen network and still maintain a functional collagen network. Further investigation will be needed to identify whether the increased collagen turnover in N_{DEG} cartilage should be considered a causal factor for the development of degeneration in these joints, *i.e.* a risk factor for OA, or whether it should be considered a consequence of the degeneration process. The latter would imply that, although the degeneration is focal, it affects cartilage metabolism in the entire joint.

EFFECT OF PROPERTIES OF THE COLLAGEN NETWORK AS IT IS LAID DOWN AT ADOLESCENCE IN THE DEVELOPMENT OF OA AT OLD AGE

Inasmuch as the ultimate effect of age-related changes in the properties of the collagen network is also determined by the quality at the start of the aging process (Figure 3), the collagen network as it is laid down at adolescence is of particular interest. During development, the cartilage collagen network may be seen as a dynamic, continuously remodeling network that is gradually taking on the biochemical characteristics it will have during the rest of the individual's life as collagen turnover is extremely slow at mature age.[204] This is illustrated by the fact that cartilage collagen modifications in the equine metacarpophalangeal joint are uniform at birth.[53] During the first months after birth, site differences in cartilage collagen hydroxylysine content start to develop

within the joint, which is likely related to the loading pattern of the cartilage.[53]

To study the role of human cartilage collagen characteristics as acquired up to adolescence (at the age of ~20 years) in the development of OA at old age, levels of the enzymatic posttranslational modifications of collagen were compared between degenerated (DG; Mankin grade 4-8) and normal cartilage from the same individual (**chapter 7**). We hypothesized that deviations in these modifications of collagen result in subtle changes that give rise to qualitative differences in the mechanical properties of the collagen network. Sample pairs were selected for comparable pentosidine levels between DG and normal cartilage. As a result, the comparison of DG and normal cartilage is not confounded by *de novo* synthesis of collagen, as occurs during cartilage degeneration[6,258] (*i.e.* the collagen network in DG cartilage reflects the situation before the onset of fibrillation).

Degenerated cartilage contained higher levels of hydroxylysine (Hyl) throughout the depth of the tissue and higher levels of pyridinoline cross-linking (HP) on the articular side (**chapter 7**). Considering the normal residence time of the collagen network, the observed changes must have been present several decades before the cartilage became degenerated. These data suggest that high levels of these enzymatic posttranslational modifications in collagen, as synthesized during the first two decades of life, result in an unfavorable quality of the collagen network at the start of the aging process. This poor "starting quality", on top of the age-related deterioration of the collagen network properties (Figure 3), results in an accelerated onset of cartilage degeneration.

CONCLUSIONS

In the present work we provide evidence for the influence of different collagen network characteristics in cartilage on the sensitivity of the tissue to become degenerated. The enzymatic posttranslational modifications of collagen as synthesized during development contribute to the quality of the collagen network at the start of the aging process, while the age-related accumulation of AGEs in collagen after maturity results in deterioration of the quality of the collagen network with aging. These combined results have led us to develop a model that explains how both processes contribute to the age of onset of OA (Figure 3). The main focus of our studies has been on the effects of the age-related accumulation of AGEs in human articular cartilage collagen and, to a lesser degree, in aggrecan. The level of AGEs is mainly determined by protein turnover. Inasmuch as the turnover of collagen in normal articular cartilage is slow (half-life >100 years),[208] relatively high AGE levels accumulate in cartilage compared to other tissues. Increasing AGE cross-linking of cartilage collagen by *in vitro* incubation with reducing sugars leads to increased stiffness and brittleness of the collagen network. Thus, the age-related accumulation of AGEs in collagen explains the reported increase in stiffness of the collagen network with age.[35,122] This may, through increased brittleness, contribute to the age-related increase in susceptibility of cartilage to degeneration. Furthermore, high AGE levels in cartilage impair the synthetic capacity of chondrocytes, which diminishes the ability of chondrocytes to restore matrix integrity after injury and may contribute to the progression of cartilage defects to OA.[82] Maintenance of matrix integrity and matrix repair may also be affected by the decreased degradation of AGE-modified cartilage proteins.[82] Proof-of-concept for the involvement of AGE accumulation in the development of OA was obtained from a study in which cartilage AGE levels in knee joints of Beagle dogs were artificially enhanced by intra-articular ribose injections. In these dogs, enhanced AGE levels resulted in increased susceptibility to develop OA after induction of joint instability by anterior cruciate ligament transection.[82,265] In addition, the fact that in humans the occurrence of focal cartilage degeneration is correlated with a high rate of AGE formation in cartilage collagen provides further evidence that

AGE cross-linking predisposes to the development of OA. The age-related accumulation of AGE cross-links in human articular cartilage collagen thus presents a molecular mechanism whereby age is a predisposing factor for the development of OA (Figure 3). Yet, the eventual result of age-related changes in cartilage collagen also depends on the "quality" of the collagen network at the start of the aging process, which is, at least partly, determined by the enzymatic posttranslational modifications of collagen as synthesized during development.

DIABETES AND OSTEOARTHRITIS

Several complications of diabetes mellitus, such as cataract, atherosclerosis, and nephropathy, resemble processes that are characteristic of aging, but these processes often occur at an earlier age in diabetes.[226] Due to the hyperglycemia, the accumulation of AGEs in long-lived extracellular proteins, also characteristic of aging, is accelerated in diabetes.[93] Furthermore, the fact that the severity of complications correlates with AGE levels[228] adds to the believe that hyperglycemia-accelerated AGE formation is one of the mechanisms involved in the genesis of late diabetic complications.

Based on our results (this thesis and Ref. 82), enhanced AGE cross-linking of cartilage collagen in diabetes is expected to contribute to an increased risk to develop OA. Consistent with what has been described for other tissues,[93] collagen AGE levels in cartilage from human diabetes patients and diabetic rats were increased compared to healthy controls (unpublished results). In a study using diabetic rats, Caterson *et al.*[67] showed increased hydration of cartilage from diabetic rats compared to normal rats. Since this swelling indicates increased damage to the collagen network,[31] these results are consistent with AGE cross-linking increasing the susceptibility of the cartilage collagen network to damage. In human studies, relatively old publications show that radiographic OA is more common, more severe, and present at a younger age in diabetic patients than in controls.[65,302,349] In recent, population based studies, only trends to positive correlations between radiographic OA and diabetes have been observed.[11,19] Plasma glucose levels have been shown to be higher in patients with radiographically confirmed symptomatic OA than in controls,[71] but this result could not be confirmed.[114] An explanation for the discrepancy between old and more recent literature may be the substantially improved glycemic control in diabetics over the past 20 years. Furthermore, in recent studies radiographic OA is assessed using the Kellgren and Lawrence grading system (which also scores the presence of osteophytes),[156] or symptomatic instead of radiographic OA is used as a measure of joint destruction. The latter two issues may result in an underestimation of the presence of OA among diabetic patients because diabetics form less osteophytes,[146] which have been suggested to be involved in the perception of pain in OA.[198,309]

Experimental studies indicate that diabetes may have a profound impact on cartilage metabolism. In streptozotocin-induced diabetic rats, mesenchymal cell proliferation, cartilage formation, synthesis of sulfated proteoglycans, and the size of proteoglycan aggregates are reduced.[67,353,354] Some of these changes may be brought about by other mechanisms than the presence of increased AGE levels. The diminished proteoglycan synthesis is suggested to be mediated by the absence of insulin, since this hormone stimulates chondrocyte biosynthesis[67,106,353] and chondrocytes express the insulin receptor.[106,317] Nevertheless, our data also showed a decrease in chondrocyte proteoglycan synthesis as a result of increased AGE cross-linking.[82]

In conclusion, studying the relation between diabetes and OA does not seem to help with a proof-of-concept that AGE accumulation is important for the development of OA. The most likely explanation for this apparent discrepancy is that diabetes is not only a disorder in which AGE levels are increased, but it is also a hormonal dysregulation that affects cartilage and its me-

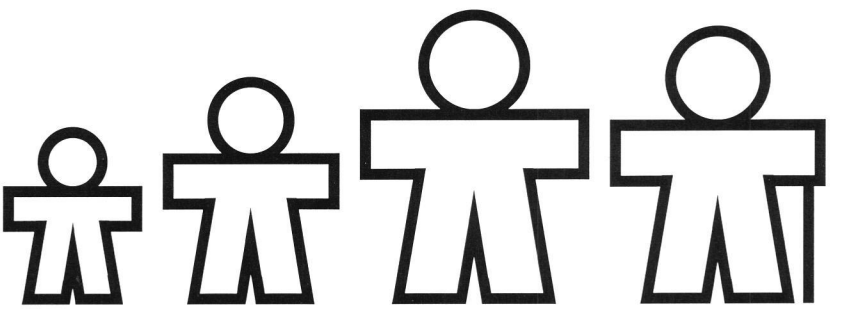
tabolism in many ways, which can be both "beneficial" or "deleterious" for the attempt of the tissue to withstand degeneration.

NEW POSSIBILITIES TO PREVENT OR DELAY THE DEVELOPMENT OF OA?

The identification of AGE accumulation as a molecular mechanism whereby age is a predisposing factor for OA development provides interesting new therapeutic possibilities to prevent OA. Our results suggest that prevention of OA could be achieved using agents that are capable of inhibiting or even reversing AGE formation. AGE formation can be inhibited by compounds such as aminoguanidine, pyridoxamine, tenilsetam, or simple amino acids (*e.g.* lysine or arginine), resulting in prevention of AGE-induced protein cross-linking, tissue collagen accumulation, and tail tendon or cardiac stiffening.[41,61,164,219,244,296,301] Furthermore, lysine and arginine effectively inhibited the AGE-induced stiffening of the cartilage collagen network in our own studies (**chapter 5**). Potentially even more exciting, agents that are able to "break" already formed AGE cross-links[335,362] may, through reversal of an increase in collagen network stiffness, slow down the development of OA. These thiazolium derivatives such as *N*-phenacylthiazolium bromide (PTB) and phenyl-4,5-dimethylthiazolium chloride (ALT-711), designed to break dicarbonyl-containing AGEs,[335] effectively reversed diabetes-induced tail tendon cross-linking and artery stiffening,[362] and the age-related increase in cardiac stiffness and accompanying impaired cardiac function.[16] However, these compounds have also been suggested to interfere with AGE formation[165,326] rather than breaking AGEs. Furthermore, a variety of AGEs is formed *in vivo* most of which have not even been characterized, making it hard to believe that chemical interference with one specific cross-link structure will be enough for effective anti-AGE therapy.

Although AGE accumulation is mainly regarded as a process that is detrimental to proteins and tissues, AGEs also seem to act as an 'age' marker of proteins and cells, which leads to their endocytosis and degradation via the scavenger receptor of *e.g.* macrophages and liver endothelial cells.[305,345] AGEs also play a role in the immune system via their interaction with RAGE, which results in cellular activation and generation of key proinflammatory mediators.[139] These functions of AGEs will have to be considered while studying the applicability of anti-AGE therapy.

Our results suggest that AGE accumulation in cartilage may contribute to the age-related increase in OA incidence by a variety of mechanisms: not only do AGEs result in increased brittleness of the cartilage collagen network which makes it more susceptible to damage, AGEs also result in a diminished capacity of chondrocytes to repair matrix damage. *In vivo* proof for the involvement of AGEs in the development OA was obtained from a canine OA model and from a cross-sectional population study. These results are the first to provide a molecular mechanism by which aging predisposes to the development of OA and suggest that anti-glycation or anti-AGE therapy may be a valuable tool to prevent or delay the onset of OA. Since most of the elderly suffer from this chronic disabling disorder, the potential use of anti-AGE therapy, as is currently being developed for diabetes, to prevent OA merits further investigation.



Appendix A

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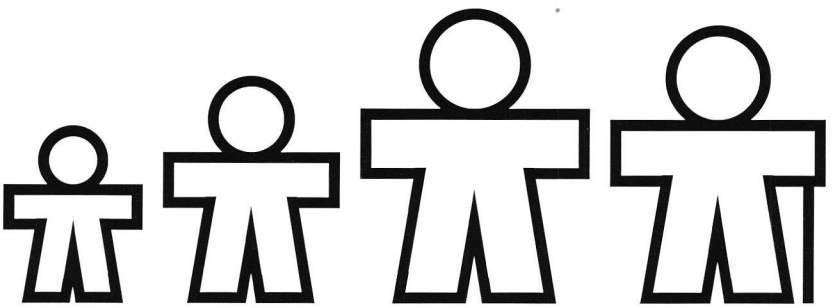
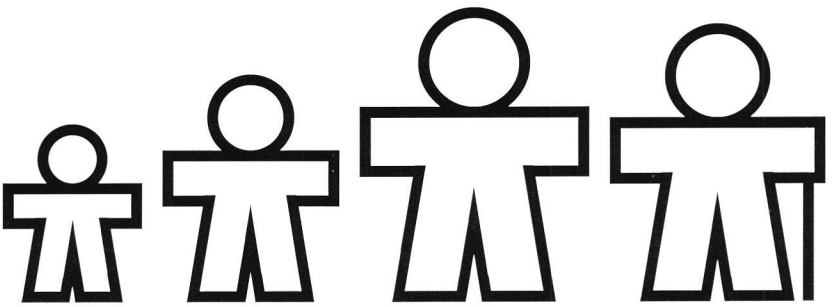


Appendix B

List of Abbreviations

A1D1	intact aggrecan monomer
AGE	advanced glycation endproduct
AGE-R1	oligosaccharyl transferase-48 (OST-48)
AGE-R2	80K-H phosphoprotein
AGE-R3	galectin-3
CEL	N^{ε}-(carboxyethyl)lysine
CML	N^{ε}-(carboxymethyl)lysine
COMP	cartilage oligomeric matrix protein
CRP	C-reactive protein
CS	chondroitin sulfate
DEG	degenerated cartilage
EGF	endothelial growth factor
ER	endoplasmatic reticulum
FCD	fixed charge density
FG1	"free" G1-domain
FL	fructose-lysine
G1	N-terminal globular hyaluronan binding domain of aggrecan
G2	globular domain of aggrecan
G3	C-terminal globular domain of aggrecan
GOLD	glyoxal-lysine dimer
HP	hydroxylysylpyridinoline
Hyl	hydroxylysine
Hyp	hydroxyproline
ID	instantaneous deformation
IGD	inter globular domain
k_{Asp}	racemization constant
k_F	formation constant
k_T	turnover constant
KS	keratan sulfate
LP	lysylpyridinoline
MOLD	methylglyoxal-lysine dimer
MMP	matrix metalloproteinase
N_{DEG}	macroscopically normal cartilage from knees with focal cartilage degeneration
NEG	nonenzymatic glycation
NFC-1	non-fluorescent cross-link 1
OA	osteoarthritis
P_c	collagen network tensile stiffness
π_{PEG}	polyethylene glycol osmotic pressure
π_{PG}	proteoglycan osmotic pressure
RAGE	receptor for AGEs
SIM-GS/MS	selected ion monitoring gas chromatography - mass spectrometry
TIMP	tissue inhibitor of matrix metalloproteinase
$V_{normalized}$	normalized tissue volume



Appendix C

Niet-Enzymatische Glycering in het Ontstaan van Artrose: Kraakbeen Biochemie en Biomechanica

Samenvatting voor niet-ingewijden

Kraakbeen is een gespecialiseerd weefsel dat zich aan het uiteinde van botten in gewrichten bevindt. Kraakbeen functioneert als een schokdemper en zorgt ervoor dat de botten een glad oppervlak hebben zodat ze soepel over elkaar kunnen bewegen. In kraakbeen zitten bijna geen cellen. Het weefsel ontleent zijn eigenschappen aan de stof tussen de cellen, de extracellulaire matrix. De extracellulaire matrix van kraakbeen bestaat voornamelijk uit collageeneiwitten, proteoglycanen en water. De collageeneiwitten vormen een driedimensioneel netwerk dat het weefsel structuur en stevigheid geeft. Binnen dit netwerk zitten proteoglycanen opgesloten. Deze proteoglycanen trekken water aan en 'zuigen' als het ware water het kraakbeen in. De uiteindelijke zwelling van het kraakbeen wordt bepaald door de rek in het collageennetwerk: de water opname stopt als het netwerk 'op spanning' is. Onbelast kraakbeen bestaat voor ongeveer 70% uit water! Als het kraakbeen belast wordt (bijvoorbeeld in de knie tijdens het zetten van een stap), wordt er water uit het kraakbeen gedrukt. Wanneer de belasting weer weg is (bijvoorbeeld bij het optillen van een voet voor de volgende stap) zorgen de proteoglycanen ervoor dat er weer water het kraakbeen ingezogen wordt. Door middel van deze waterverplaatsingen kan het kraakbeen werken als een schokdemper. Artrose (in het Engels: Osteoarthritis), of 'kraakbeenslijtage', is een veel voorkomende chronische aandoening die uiteindelijk kan leiden tot destructie van het gewricht. Voor de patiënt betekent artrose vooral pijn in gewrichten en beperking van de beweeglijkheid. In ernstige gevallen kan dit zelfs leiden tot invaliditeit. Bij jonge mensen komt artrose nauwelijks voor, maar het aantal mensen met artrose neemt sterk toe bij toenemende leeftijd: meer dan 60% van de 60+-ers heeft een vorm van artrose. Lang voordat iemand, als gevolg van artrose, met pijn bij de dokter komt zijn er in het kraakbeen zelf al veranderingen waar te nemen. De vroegste verandering die in het kraakbeen wordt waargenomen bij het ontstaan van artrose is beschadiging van het collageennetwerk.

Suikers spelen een belangrijke rol als bouwstof in het lichaam en zijn van belang voor de energievoorziening. Daarom zijn suikers onmisbaar voor ieder levend organisme. De meeste processen in ons lichaam waarbij suikers betrokken zijn worden sterk gecontroleerd en gereguleerd door enzymen (de "reactieversnellers" van ons lichaam). Toch blijken suikers ook een rol te spelen in een proces dat spontaan verloopt en weinig nuttige functies lijkt te hebben: de niet-enzymatische glycering.

Niet-enzymatische glycering is de spontane reactie van suikers, zoals glucose, met de bouwstenen van eiwitten (aminozuren). In essentie zijn dit eenvoudige chemische reacties; hoe snel de reactie verloopt wordt voornamelijk bepaald door de hoeveelheid suiker en aminozuren die aanwezig zijn en de temperatuur. De aminozuren lysine, hydroxylysine en arginine zijn met name gevoelig voor deze niet-enzymatische reactie met suikers. Nadat een verbinding is ontstaan tussen de suiker en het aminozuur kunnen in vervolgreacties een heleboel verschillende producten ontstaan waaronder dwarsverbindingen (cross-links) tussen eiwitten. Eén van de bekendste en best gekarakteriseerde cross-links is door de ontdekkers 'pentosidine' genoemd. De vorming van deze glyceringsproducten (in het Engels: Advanced Glycation Endproducts) verloopt spontaan. Het lichaam heeft geen manier om deze producten te verwijderen anders dan het hele eiwit af te breken waarin die producten gevormd zijn. Als een eiwit lang in ons lichaam aanwezig blijft worden er dus steeds nieuwe glyceringsproducten in gevormd die als het ware opstapelen in het

eiwit. Hierdoor kan de hoeveelheid glyceringsproducten in eiwitten met een lage "vernieuwingssnelheid" sterk toenemen met toenemende leeftijd. Het collageen in kraakbeen is zo'n eiwit. Tijdens de groei (tot de leeftijd van 20 jaar) wordt het collageen in kraakbeen regelmatig vernieuwd. Daardoor worden gevormde glyceringsproducten in deze periode nog regelmatig verwijderd. Echter, na het bereiken van volwassenheid is er bijna geen vernieuwing meer van het kraakbeen collageen. Daardoor zal in het kraakbeencollageen van volwassenen de hoeveelheid glyceringsproducten snel toenemen. Hierdoor veranderen de eigenschappen van het collageennetwerk.

Alhoewel leeftijd een belangrijke risicofactor is voor artrose is het nog niet duidelijk hoe een hoge leeftijd kan bijdragen aan de verhoogde kans op artrose. De leeftijdsgerelateerde toename in artrose gaat gelijk op met de toename in glycering van het collageen in gewrichtskraakbeen. Dit suggereert een verband tussen glycering van kraakbeen collageen en artrose. Daarbij komt nog dat schade aan het collageennetwerk de eerste stap lijkt in het ontstaan van artrose. Onze studies (beschreven in dit proefschrift en in het proefschrift van Jeroen de Groot: 'Advanced glycation endproducts in the development of osteoarthritis: cartilage synthesis and degradation') hebben zich gericht op de vraag of niet-enzymatische glycering van kraakbeen ertoe bijdraagt dat leeftijd een belangrijke risicofactor is voor het ontstaan van artrose. Deze studies gaan uit van het idee dat de cross-links die ontstaan als gevolg van niet-enzymatische glycering leiden tot het stijver worden van het collageennetwerk in kraakbeen: het netwerk wordt als het ware meer-en-meer aan elkaar geknoopt, waardoor het uiteindelijk broos wordt. Dit broze collageennetwerk kan makkelijk beschadigd raken en het begin zijn van het ontstaan van artrose.

Uit werk van andere onderzoekers is bekend dat kraakbeen, vergeleken met andere weefsels zoals de huid, relatief veel van het glyceringsproduct pentosidine bevat. Pentosidine is slechts één van de producten die kan ontstaan als gevolg van niet-enzymatische glycering. Toch suggereert de aanwezigheid van veel pentosidine in kraakbeen dat kraakbeen in het algemeen veel glyceringsproducten zou kunnen bevatten. In **hoofdstuk 2** van dit proefschrift is voor een aantal bekende glyceringsproducten beschreven in welke mate ze met toenemende leeftijd opstapelen in kraakbeencollageen. Tevens is de hoeveelheid glyceringsproducten in kraakbeencollageen vergeleken met collageen in de huid en langlevende eiwitten in de ooglens. Uit deze vergelijking bleek dat kraakbeencollageen inderdaad veel glyceringsproducten bevat. Het meest opvallende resultaat was dat in kraakbeencollageen de meetbare hoeveelheid van de aminozuren die gevoelig zijn voor glycering (arginine, hydroxylysine en lysine) afneemt met de leeftijd. Dit komt doordat een aminozuur met onze methode niet meer gemeten kan worden nadat een suiker ermee heeft gereageerd. Deze afname in hoeveelheid aminozuren met de leeftijd - een heel algemene maat voor niet-enzymatische glycering - wordt niet gezien in huidcollageen en lenseiwitten.

Zoals ook hierboven al aangehaald is, wordt heel vaak gezegd dat de toename in de hoeveelheid glyceringsproducten in een eiwit afhangt van de vernieuwingssnelheid van dat eiwit. Echter, tot nu toe heeft niemand dit verband daadwerkelijk aangetoond. Voor onze studie hebben we gebruik gemaakt van een methode waarmee de 'leeftijd' van een eiwit gemeten kan worden, los van de leeftijd van het individu waarvan dit eiwit afkomstig is. In **hoofdstuk 3** zijn de resultaten beschreven die laten zien dat kraakbeen collageen drie keer meer glyceringsproducten bevat dan huid collageen, ongeacht de leeftijd van het individu. Op de maat voor eiwitleeftijd scoorde kraakbeen collageen ook drie keer zo hoog als het collageen in de huid. Deze resultaten geven aan dat het verschil in de hoeveelheid glyceringsproducten tussen kraakbeen en huid collageen terug te voeren is op het verschil in 'leeftijd' van deze collageen eiwitten. Uit

onze meting van de eiwitleeftijd hebben we ook kunnen berekenen hoe snel kraakbeen en huid collageen vernieuwen in ons lichaam: in kraakbeen is de helft van het collageen vervangen na 117 jaar, in de huid al na 14 jaar. Voor kraakbeen betekent dit dat de meerderheid (65-70%) van het collageen dat in het weefsel is neergelegd als we 20 jaar oud zijn nog steeds aanwezig is in ons kraakbeen als we 70 zijn!

Omdat schade aan het collageennetwerk in kraakbeen het eerste teken is van het ontstaan van artrose heeft ons onderzoek zich vooral gericht op het collageen in kraakbeen. Het grootste deel van de cross-link pentosidine in kraakbeen is ook aanwezig in het collageen (80-85%). Zoals boven beschreven zijn de proteoglycanen het andere belangrijke vaste bestanddeel van de kraakbeen matrix. Glyceringsproducten blijken ook met de leeftijd toe te nemen in de proteoglycaanmoleculen in kraakbeen, vooral in het proteoglycaan dat aggrecan heet. Deze resultaten zijn in **hoofdstuk 4** beschreven. Aggrecan heeft een hogere vernieuwingssnelheid dan collageen, oftewel: het wordt minder oud. Daardoor was het niet verwonderlijk dat we vonden dat de hoeveelheid pentosidine in aggrecan lager is dan in collageen bij een zelfde leeftijd van het kraakbeen. Binnen de aggrecanmoleculen in kraakbeen bestaan er ook weer grote verschillen in leeftijd en de oudste moleculen bleken de meeste pentosidine te bevatten. Dit klopt met onze andere gegevens (hoofdstuk 3) dat de hoeveelheid glyceringsproducten sterk afhangt van de leeftijd van het eiwit. In de bijlage van hoofdstuk 4 hebben we zelfs laten zien dat de hoeveelheid pentosidine in aggrecan gebruikt kan worden om de leeftijd van het eiwit te berekenen.

Het is bekend dat de vernieuwingssnelheid van aggrecan omhoog gaat tijdens artrose; op deze manier proberen de cellen in het kraakbeen de schade die ontstaat aan het weefsel te herstellen. Het nieuwe aggrecan dat aangemaakt wordt in het artrotische kraakbeen bevat nog helemaal geen glyceringsproducten (die zijn nog niet gevormd). Daarom verwachtten we dat de hoeveelheid pentosidine in artrotisch kraakbeen lager zal zijn dan in gezond kraakbeen en dit hebben we ook gemeten: in aggrecan dat opgezuiverd was uit artrotisch kraakbeen zat minder pentosidine dan in aggrecan uit gezond kraakbeen (hoofdstuk 4).

Zoals gezegd gingen we uit van het idee dat de cross-links die door niet-enzymatische glycering kunnen ontstaan ervoor zorgen dat het collageennetwerk in kraakbeen stijver wordt. Door de toegenomen stijfheid zou het collageennetwerk 'brozer' kunnen worden en dus gevoeliger voor beschadiging; het begin van artrose. In **hoofdstuk 5** hebben we getest of niet-enzymatische glycering inderdaad zorgt voor het stijver worden van het collageennetwerk. Om dit te testen hebben we kraakbeen van jonge mensen blootgesteld aan hoge suikerconcentraties zodat kunstmatig extra veel glyceringsproducten in het kraakbeen gevormd worden. De stijfheid van het kraakbeen hebben we gemeten door op stukjes kraakbeen te drukken (in dezelfde richting als waarin dat in een gewricht gebeurt) en te meten hoeveel het kraakbeen vervormde onder deze belasting; hoe minder vervorming, hoe stijver het collageennetwerk in het kraakbeen. Na reactie van threose (een erg reactieve suiker) met het kraakbeen bleek de hoeveelheid glyceringsproducten in het collageen sterk toe te nemen. De stijfheid van het collageennetwerk nam ook sterk toe na de behandeling met deze suiker en ging gelijk op met de toename in glyceringsproducten. Wanneer tijdens de behandeling met threose ook remmers van niet-enzymatische glycering aanwezig waren kon de toename in stijfheid voorkomen worden. In een ander soort experiment is bovendien gebleken dat behandeling met suiker ervoor zorgt dat het collageennetwerk in kraakbeen makkelijker kapot gaat wanneer er aan het kraakbeen getrokken wordt. Kortom, niet-enzymatische glycerings cross-links zorgen ervoor dat het collageennetwerk in kraakbeen stijver én brozer wordt. Het feit dat kraakbeen met toenemende leeftijd steeds meer glycerings-

producten gaat bevatten kan dus bijdragen aan een toename in gevoeligheid voor beschadiging met de leeftijd, en daarmee voor het ontstaan van artrose.

Ervan uitgaand dat niet-enzymatische glycering een rol speelt bij het ontstaan van artrose zou het zo kunnen zijn dat het kraakbeencollageen van mensen die artrose krijgen meer glyceringsproducten bevat dan het kraakbeencollageen van mensen die geen artrose krijgen. Omdat er tijdens het artrose proces erg veel verandert in het kraakbeenweefsel hebben we, om bovenstaande vraag te onderzoeken, alleen gebruik gemaakt van kraakbeen uit artrose gewrichten dat op het oog nog volledig normaal was. Dit kraakbeen hebben we vergeleken met het kraakbeen van mensen die alleen maar gezond kraakbeen hadden. Uit de resultaten die beschreven zijn in hoofdstukken 3 en 4 weten we dat verschillen in vernieuwingssnelheid van het collageen leiden tot verschillen in de hoeveelheid glyceringsproducten. Daarom hebben we in deze studie (**hoofdstuk 6**) de gemeten hoeveelheid glyceringsproducten in het kraakbeen gecorrigeerd voor de leeftijd van het collageen. Door deze correctie toe te passen wordt als het ware de 'glyceringssnelheid' gemeten. Uit de resultaten bleek dat de glyceringssnelheid hoger is in gewrichten met artrose dan in gezonde gewrichten. Dit duidt op een verhoogde gevoeligheid om artrose te krijgen in mensen waarin glyceringsproducten sneller ontstaan. Uit onze metingen van de leeftijd van het collageen bleek tevens dat het normale kraakbeen van de gewrichten met artrose relatief meer jong collageen bevat dan het kraakbeen uit gezonde gewrichten. Dit jonge collageen was intact en normaal ingebouwd in het collageennetwerk. Wetenschappers gaan er over het algemeen van uit dat in volwassen kraakbeen nauwelijks nieuwe aanmaak van collageen optreedt. Onze resultaten laten zien dat dit wél gebeurt en dat het leidt tot een normaal collageennetwerk.

Tot nu toe hebben we ons vooral gericht op leeftijdsgerelateerde veranderingen in volwassen kraakbeen (vanaf 20-jarige leeftijd). Toch wordt het eindresultaat van veranderingen in de kwaliteit van het kraakbeen met toenemende leeftijd ook bepaald door het beginpunt: de kwaliteit van het kraakbeen aan het eind van de ontwikkeling naar volwassenheid. Dit is vooral belangrijk omdat het collageennetwerk in volwassen kraakbeen een langzame vernieuwingssnelheid heeft en dus weinig mogelijkheden heeft om zich aan veranderde omstandigheden aan te passen. Om dit te onderzoeken hebben we eigenschappen van het collageennetwerk, anders dan glycering, bestudeerd in aangedaan en gezond kraakbeen van hetzelfde individu. We hebben voor deze vergelijking alleen aangedaan en gezond kraakbeen gebruikt waarin het collageennetwerk van een vergelijkbare leeftijd was. Door deze restrictie worden veranderingen die als gevolg van het ziekteproces ontstaan in het collageennetwerk in het aangedane kraakbeen uitgesloten. Het bleek dat in collageen in aangedaan kraakbeen meer van bepaalde enzymatisch aangelegde collageenstructuren aanwezig waren dan in het gezonde kraakbeen (**hoofdstuk 7**). De verhoogde aanwezigheid van die structuren kan leiden tot een subtiele verslechtering van de kwaliteit van het collageennetwerk bij het bereiken van volwassenheid. Op latere leeftijd kan dat bijdragen aan het ontstaan van artrose. Hoe deze eigenschappen van het collageen samen met de leeftijdgerelateerde verslechtering van de kwaliteit van het collageen in kraakbeen (door glycering) zouden kunnen bepalen op welke leeftijd iemand artrose krijgt is weergegeven in het model in figuur 3 in hoofdstuk 8.

Samengevat concluderen wij dat glycering mogelijk een belangrijke bijdrage levert aan het ontstaan van artrose door verhoogde cross-linking van het collageennetwerk, met als gevolg dat het collageennetwerk in kraakbeen stijver wordt. Dit leidt tot brozer weefsel dat bij normale belasting sneller schade zal oplopen: een eerste stap in het ontstaan van artrose. Naast effecten op de stijfheid van het kraakbeen, levert glycering mogelijk ook een bijdrage aan het ontstaan van artrose doordat het de mogelijkheid van

de cellen in het kraakbeen om matrix aan te maken en af te breken vermindert (zie proefschrift Jeroen de Groot; hoofdstukken 2-5). Deze eigenschappen zijn nodig om schade aan het kraakbeen te kunnen herstellen. In geglyceerd kraakbeen kan schade aan de kraakbeen matrix langzamer hersteld worden door de kraakbeencellen. Dat kan er voor zorgen dat een klein defect, dat in niet-geglyceerd kraakbeen hersteld had kunnen worden, uiteindelijk toch leidt tot het ontstaan van artrose. Daarnaast kan toegenomen glycering, door vermindering van de aanmaak en afbraak van kraakbeen matrix, ervoor zorgen dat het kraakbeen niet snel genoeg aangepast wordt aan veranderde omgevingsfactoren (zoals bijvoorbeeld een verandering in belasting van het kraakbeen). Minder goed aangepast kraakbeen zal sneller beschadigen. In een dierexperimenteel onderzoek (proefschrift Jeroen de Groot; hoofdstuk 6) is aangetoond dat verhoogde glycering inderdaad leidt tot versnelde ontwikkeling van artrose. Daarnaast blijken mensen die artrose ontwikkelen sneller glyceringsproducten te vormen dan mensen zonder artrose. Dus, de toename in glycering van kraakbeen met de leeftijd lijkt - tenminste ten dele - te kunnen verklaren waarom het risico op artrose toeneemt met de leeftijd. Daarnaast hangt het effect van leeftijdsgerelateerde veranderingen ook af van de kwaliteit van het kraakbeen collageennetwerk zoals het is aangelegd tijdens de ontwikkeling tot volwassenheid.

Glycering is niet alleen betrokken bij verouderingsziekten. Het is ook betrokken bij het ontstaan van complicaties van suikerziekte (diabetes mellitus). Diabetes patiënten hebben jarenlang met een zekere regelmaat te veel suiker in hun bloed waardoor de eiwitten in hun lichaam aan hogere suikerconcentraties worden blootgesteld. Dit resulteert al op jonge leeftijd in de vorming van relatief veel glyceringsproducten in lichaamseiwitten wat leidt tot het vervroegd optreden van verouderingsverschijnselen in diabetes patienten.

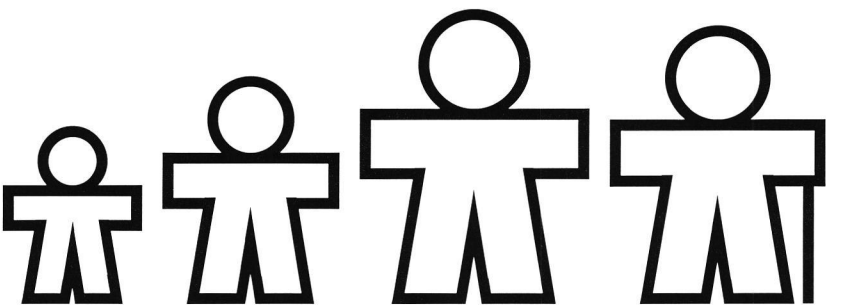
Oudere onderzoeken (gepubliceerd rond 1960) laten zien dat mensen met diabetes op jongere leeftijd artrose krijgen dan niet-diabeten en dat diabetes patiënten vaak ernstigere artrose krijgen. Deze gegevens sluiten mooi aan bij ons vermoeden dat niet-enzymatische glycering - hoger bij diabetes patiënten - een rol speelt bij het ontstaan van artrose. In recenter onderzoek wordt dit verband vaak niet meer gevonden. Eén van de oorzaken hiervoor zou kunnen zijn dat de controle op suikerconcentraties in het bloed van diabetes patiënten sterk verbeterd is.

Het moge duidelijk zijn dat glycering niet levensbedreiging nummer één is. De kwaliteit van leven op oudere leeftijd en bij diabetes kan er echter wel negatief door beïnvloed worden. Het voorkómen dan wel ongedaan maken van glyceringsproducten zou dus bij kunnen dragen aan verbetering van de kwaliteit van leven van ouderen. Onderzoek is in eerste instantie voornamelijk gericht geweest op mogelijkheden om de vorming van glyceringsproducten te remmen. Hiertoe is bijvoorbeeld de effectiviteit van de aminozuren lysine en arginine getest om de vorming van glyceringsproducten in diermodellen voor diabetes te remmen. Alhoewel soms duidelijk effecten te zien waren, lijkt het erg moeilijk om verouderingsgerelateerde opstapeling van glyceringsproducten op deze manier te verlagen: mensen zouden deze aminozuren jarenlang moeten slikken om voldoende effect te bewerkstelligen.

De alternatieve aanpak, het teniet doen van al gevormde glyceringsproducten, heeft daarom recent meer aandacht gekregen. Er zijn nu stoffen beschreven die cross-links kunnen verbreken die ontstaan zijn als gevolg van niet-enzymatische glycering. Deze aanpak bleek de toegenomen stijfheid van vaatwanden in dieren met diabetes weer normaal te maken. Ook waren deze stoffen in staat de stijfheid van hartweefsel in oude dieren te verlagen zodat de hartfunctie weer meer op die van jonge dieren ging lijken. Als deze weg met succes wordt ingeslagen zullen mogelijk in de toekomst de negatieve, ongecontroleerde glyceringseffecten van

suikers (die als brandstof in het lichaam van levensbelang zijn) tenietgedaan kunnen worden.

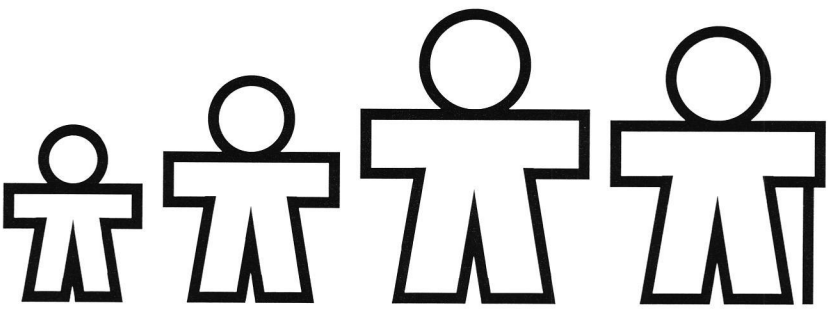
Deze bevindingen samen met onze resultaten duiden erop dat therapieën die gericht zijn op het tegengaan en/of ongedaan maken van niet-enzymatische glycering een belangrijke bijdrage kunnen leveren aan preventie en behandeling van artrose en daarmee aan het verhogen van de kwaliteit van leven van ouderen.



Appendix D

List of Publications

1. Beekman B, Verzijl N, De Roos JADM, Koopman JL and TeKoppele JM (1997) Doxycycline inhibits collagen synthesis by bovine chondrocytes cultured in alginate. *Biochem. Biophys. Res. Comm.* 237: 107-110.
2. Beekman B, Verzijl N, Bank RA, Von Der Mark K and TeKoppele JM (1997) Synthesis of collagen by bovine chondrocytes cultured in alginate: posttranslational modifications and cell-matrix interactions. *Exp. Cell Res.* 237: 135-141.
3. Bank RA, Beekman B, Verzijl N, De Roos JADM, Sakkee AN and TeKoppele JM (1997) Sensitive fluorimetric quantitation of pyridinium and pentosidine crosslinks in biological samples using a single high-performance liquid chromatographic run. *J. Chromatography B Biomed. Sci. Appl.* 703: 37-44.
4. TeKoppele JM, Beekman B, Verzijl N, Koopman JL, DeGroot J and Bank RA (1998) Doxycycline inhibits collagen synthesis by differentiated articular chondrocytes. *Adv. Dental Res.* 12: 63-67.
5. Schalkwijk CG, Vermeer MA, Verzijl N, Stehouwer CDA, TeKoppele JM, Princen HMG and Van Hinsbergh VWM (1998) Modification of low-density lipoprotein by methylglyoxal alters its physico-chemical and biological properties. In: O'Brien J, Nursten HN, Crabbe MJC and Ames JM, editors. *The Maillard reaction in foods and medicine.* Royal Society of Chemistry (Cambridge), pp. 285-290.
6. Beekman B, Verzijl N, De Roos JADM and TeKoppele JM (1998) Matrix degradation by chondrocytes cultured in alginate: IL-1ß induces proteoglycan degradation and proMMP synthesis but does not result in collagen degradation. *Osteoarthritis Cartilage* 6: 330-340.
7. Handa JT, Verzijl N, Matsunaga H, Aotaki-Keen A, Lutty GA, Te Koppele JM, Miyata T and Hjelmeland LM (1999) Increase of the advanced glycation endproduct pentosidine in Bruch's membrane with age. *Invest. Ophthalmol. Vis. Sci.* 40: 775-779.
8. DeGroot J, Verzijl N, Bank RA, Lafeber FPJG, Bijlsma JWJ and TeKoppele JM (1999) Age-related decrease in proteoglycan synthesis of human articular chondrocytes: the role of nonenzymatic glycation. *Arthritis Rheum.* 42: 1003-1009.
9. Verzijl N, DeGroot J, Oldehinkel E, Bank RA, Thorpe SR, Baynes JW, Bayliss MT, Bijlsma JWJ, Lafeber FPJG and TeKoppele JM (2000) Age-related accumulation of Maillard reaction products in human articular cartilage collagen. *Biochem. J.* 350: 381-387.
10. Verzijl N, DeGroot J, Thorpe SR, Bank RA, Shaw JN, Lyons TJ, Bijlsma JWJ, Lafeber FPJG, Baynes JW and TeKoppele JM (2000) Effect of collagen turnover on the accumulation of advanced glycation endproducts. *J. Biol. Chem.* 275: 39027-39031.
11. DeGroot J, Verzijl N, Budde M, Bijlsma JWJ, Lafeber FPJG and TeKoppele JM. Accumulation of advanced glycation endproducts decreases collagen turnover by bovine chondrocytes. *Exp. Cell Res.* (in press).



Appendix E

NAWOORD

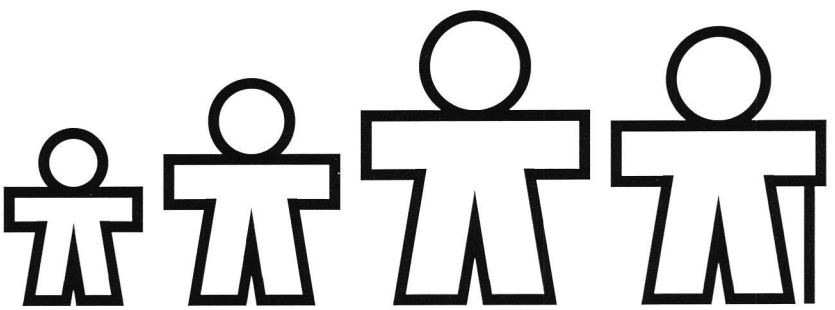
Ik zal niet de eerste promovendus zijn die op dit punt moet toegeven dat ik het onderzoek niet alleen heb gedaan. Dat had ook nooit gekund! Een groot aantal mensen heeft daar in de afgelopen jaren een bijdrage aan geleverd.

Mijn 'promotie-team', bestaande uit Johan, Floris en Hans, en mijn collega 'twAIO' Jeroen (de Groot) wil ik bedanken voor de stimulerende discussies en de team-geest waarin dit boekje tot stand is gebracht. Johan, dankjewel voor je vertrouwen en je tomeloze 'drive'; het was onmogelijk niet door jouw enthousiasme aangestoken te worden. Heerlijk hoe jij steevast geloofde in de goede afloop. Floris, ook al gaf je zelf soms aan iets verder van mijn werk af te staan, jouw kritische kijk op mijn werk heeft menig oneffenheidje eruit doen verdwijnen, dankjewel daarvoor. Hans, onze tweemaandelijkse besprekingen waren erg nuttig om de grote lijn niet uit het oog te verliezen. Bedankt ook voor het hoge tempo waarin je de afgelopen maanden stukken hebt gelezen. Jeroen (de Groot), het was best een avontuur om samen aan dit twAIO ('twee AIO's') project te beginnen. Onze gesprekken over AIO-perikelen hebben waarschijnlijk net zo'n belangrijke bijdrage geleverd aan het slagen van onze missie als onze veelvuldige inhoudelijke discussies.

Dank zij mijn (oud-)collega's en de studenten op het lab bij TNO en in Utrecht heb ik mijn onderzoek met veel plezier kunnen doen. De lol die vaak gepaard ging met daverende lachsalvo's maakte lange werkweken gezellig en haalbaar.

Alice, Susan and John, thank you all very much for your tremendous hospitality. I really enjoyed my visits to your labs, from both a scientific and a personal point of view.

Lisette en Nico, bedankt dat jullie me tijdens de verdediging als paranimfen terzijde willen staan. Meneer Polman (Emmauscollege, Rotterdam), dank u wel voor het volhardende speurwerk waarmee u het Griekse citaat boven water heeft gehaald. Papa en mama, jullie hebben altijd een rotsvast vertrouwen in mij gehad en me gestimuleerd om uit mezelf te halen wat erin zit: dank jullie wel daarvoor. Zie hier een (deel van) het resultaat. Jeroen, last-but-not-least, dank zij jou zien mijn boekje - en mijn leven - er mooi uit!



Appendix F

CURRICULUM VITAE

Nicole Verzijl was born on 20 October 1971 in Maasdam. In 1989 she finished her secondary school education at the 'Emmauscollege' in Rotterdam. From 1989 until 1995 she studied Biomedical Sciences at the University of Leiden, finishing her masters degree *cum laude*. During this study, she obtained research experience at the Department of Physiology, University of Leiden (supervisors Dr. E. Marani and Prof. W.J. Rietveld), the Department of Rheumatology, University Hospital Leiden (work performed at the Institute for Ageing and Vascular Research-TNO, Leiden; supervisors Dr. J.M. te Koppele and Prof. F.C. Breedveld), the Department of Anthropogenetics, Sylvius Laboratory, University of Leiden (supervisor Dr. D.J.M. Peters) and the Division of Vascular- and Connective Tissue Research, TNO Prevention and Health, Leiden (supervisors B. Beekman and Dr. J.M. te Koppele).

Thereafter, she worked from September 1995 until June 1996 at the Department of Immunology, Erasmus University Rotterdam. In July 1996 she started her PhD at the Department of Rheumatology & Clinical Immunology of the University Medical Center Utrecht and the Gaubius Laboratory of TNO Prevention and Health in Leiden, under the supervision of Dr. J.M. te Koppele, Dr. F.P.J.G. Lafeber and Prof. J.W.J. Bijlsma. Parts of the studies described in this thesis were performed in the laboratories of Prof. A. Maroudas (Department of Biomedical Engineering, Technion, Haifa, Israel) and Prof. J.W. Baynes (Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC, U.S.A.). Since December 2000 she continued her work at TNO Prevention and Health as group leader of the Matrix Biology research group in the Division of Immunological and Infectious Diseases.