Age and osteoarthritis: are AGEs the link?

Leeftijd en artrose: vormt glycering de link?
(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General introduction
Osteoarthritis

Osteoarthritis (OA) is a slowly developing joint disorder, characterized by progressive cartilage damage and loss, changes in bone and peri-articular soft tissues, and joint inflammation. In most cases OA is diagnosed in a late stage of the disease, due to lack of early markers. Diagnosis of OA is typically based on clinical symptoms and physical examination, sometimes with radiographic evaluation. When clinically evident, OA is characterized by joint pain, tenderness, limitations of movement, crepitus, occasional effusion, and local inflammation secondary to the disease process. OA predominantly affects the knees, hips, hands, and spine, but can affect in principle every synovial joint. Although there is no known cure for OA, treatment tailored to the needs of the individual patient can reduce pain, maintain and/or improve joint mobility, and limit functional impairment. Eventually, OA may lead to joint replacement or severe functional impairment, because of lack of treatment to cure the disease.

Epidemiology

OA is the most common disorder of the locomotor system and can affect all synovial joints. Symptomatic knee OA affects roughly 5-24% of the adult population and is the most common form of OA. Around 1-7% of the adult population is affected by hip OA. The incidence of OA steeply rises with age. As the population is aging with older people still preferring an active life style, the prevalence and burden of OA is projected to increase even more which results in huge public health consequences.

Risk factors

Although the exact pathogenesis of OA is still unclear, it is generally appreciated that its etiology is multi-factorial, with genetic, biochemical, and mechanical factors playing a role. Risk factors can be divided in general factors (e.g. age, genetics, gender, and obesity) and local factors (e.g. joint trauma, anatomical deformation, and extensive loading). Besides (known or unknown) trauma to the joint or adjacent areas or structures, aging and overloading are the main risk factors. Previous trauma to the joint such as meniscal tear, ligament insufficiency, cartilage defect, or intra-articular fracture, inevitably results in a certain degree of joint degeneration. A change in mechanical (e.g. malalignment, muscle weakness) or biochemical (e.g. inflammation) or peri-articular environment may lead to crossing a certain point of no return in tissue degeneration (a disturbed balance between tissue synthesis and breakdown) and with that inevitable initiation of OA. Also congenital joint deformities (e.g. hip dysplasia) may predispose and eventually lead to osteoarthritic changes. Biochemical factor may include acquired or inherited aberrations in activity of all cells and tissues of the joint (e.g. elevated IL-1 production by synoviocytes) and even peripheral (e.g. adipokine release in case of obesity). In all cases, a combination of biochemical and biomechanical factors are expected to play a role in development and progression of the disease. Despite multiple etiologies, the ultimate joint changes in OA are roughly considered relatively uniform (‘final common pathway’), although the identification of specific phenotypes with specific sequences of characteristics becomes more and more fashionable.

Clinical symptoms

Clinical symptoms of OA include pain, stiffness, and a diminished range of motion; eventually leading to joint dysfunction. Pain is described as relatively continuous and nagging, but is typically most severe at the initiation of activities and has commonly been present for years when the disease is diagnosed. Joint stiffening occurs especially in the morning or after other long periods of immobilization. Eventually, range of motion in the joint decreases due to pain or mechanical blocking. In more advanced stages joint shape may
be altered by adaptive changes in the surrounding bone\textsuperscript{10}. Flexion is accompanied by crepitus and there is muscle atrophy due to disuse. Diagnosis of hip or knee OA is based either solely on clinical findings or on joint pain in combination with radiographic joint damage\textsuperscript{2,11}.

**Tissues involved**

Joints are part of the bony skeleton and allow movement within the rigid pillars of our body. The range of movement of joints depends on their specific shape. The two bony ends forming the joint are covered with hyaline cartilage. The majority of joints with hyaline cartilage are synovial joints characterized by the presence of a capsule with synovial lining on the inside and a thin layer of synovial fluid within the joint space. Ligaments keep the bone ends together and muscles allow movement.

**Cartilage**

Articular cartilage is a highly specialized connective tissue that covers the ends of bones within synovial joints. This articular cartilage enables smooth movement of synovial joints, supported by the lubricating synovial fluid, and is capable of absorbing stresses by deforming under mechanical loading and thereby minimizing peak stresses on subchondral bone\textsuperscript{7}. Articular cartilage is avascular and aneural, depending mostly on the synovial fluid for its nutrition.

Articular cartilage exists of chondrocytes in an extra-cellular matrix. Water is the major component of the extra-cellular matrix (68-85\% of the weight in human cartilage). Other components are collagen type II (10-20\% of the wet weight) and proteoglycans (5-10\% of the wet weight). These three components together are responsible for the mechanical properties of cartilage. The collagen network forms the shape and tensile strength of cartilage. The proteoglycans form large aggrecan molecules within the matrix and are responsible for the resilience of the cartilage. These negatively charged proteoglycans are fixed in the matrix and are drawing cations into the matrix to balance the negative charge of the proteoglycans. This creates an osmotic potential absorbing water into the tissue, which results in a large swelling force. In the unloaded condition, swelling of the tissue is constrained by tensile stiffness of the collagen network\textsuperscript{7}. During compressive loading of the cartilage the water is squeezed out. When the cartilage is unloaded this water is absorbed again because of the osmotic pressure of the cations within the matrix, thereby providing the unique resilience of articular cartilage\textsuperscript{12}. The matrix, where the turnover of proteoglycans is much higher than that of collagen, is maintained by chondrocyte activity. The principal collagen, type II collagen, accounts for 90 to 95 percent of the collagen in articular cartilage\textsuperscript{13}. Aggrecan molecules accounts for 90\% of the volume of cartilage determining the negative charge of the matrix whereas numerous smaller proteoglycans with mostly undefined properties exceed in molecular numbers\textsuperscript{7}.

In healthy individuals, normal cartilage-matrix turnover exists in which synthesis and degradation are balanced. Chondrocytes respond to changes in the extracellular matrix, such as presence of fragmented matrix molecules, the presence of cytokines and growth factors with both catabolic and anabolic effects, and the frequency and intensity of joint loading\textsuperscript{14}. In osteoarthritis there is a progressive loss of articular cartilage accompanied by an attempt to repair. Swelling is one of the first features in cartilage degeneration, indicating collagen damage\textsuperscript{15}. In turn this leads to a loss of tensile properties and to a loss of proteoglycans. Aging causes a decrease of the capacity of the cells to synthesize some types of proteoglycans. In addition, their response to stimuli, including growth factors, decreases\textsuperscript{16}. These age-related changes may limit the ability of the cells to maintain tissue integrity and thereby contribute to the development of degeneration of the articular cartilage.
**Subchondral bone**

Subchondral bone consists of a cortical bone plate which is supported by the cancellous bone, a network of bone trabeculae, which architecture is responsible for its strength and capacity to withstand loading forces\(^{17}\). Subchondral bone changes are a distinctive feature in OA development, and they include sclerosis, cyst formation, bone marrow lesions (evidenced by MRI or CT), bone attrition, and osteophytes\(^{9}\).

One of the most remarkable and consistent features of joints affected by OA is the development of osteophytes, osteochondral nodules at the joint edges\(^{18}\). Although the exact functional significance of osteophyte growth remains unclear, osteophytes might help to stabilize joints affected by OA, however this is disputed\(^{19}\). Nowadays, osteophytes are more and more considered to be related to synovial activity (inflammation)\(^{20}\).

Subchondral bone changes beneath weight-bearing joint surface are commonly referred to as subchondral sclerosis. On radiographic imaging this reflects an increase in apparent bone density. The density of subchondral bone seems to be increased in OA because of the increased number of trabeculae and reduced separation between trabeculae, while the mineralization of the bone is significantly lower than in controls. The reduction in mineral can be explained by the increased turnover rate in osteoarthritic bone, which results in younger, less mineralized bone. It is commonly accepted that the trabecular bone and the subchondral plate each respond differently and should be approached as separate structures\(^{22-23}\). MRI studies have shown that increased bone density (sclerosis) coinciding with excessive loading, is associated with bone marrow lesions\(^{24}\). These MRI-apparent lesions are marked by bone marrow necrosis, fibrosis, and trabecular abnormalities\(^{25}\). Bone marrow lesions may play a role in the pathogenesis of subchondral cysts, as cysts have been observed to arise within regions of marrow edema-like signal\(^{26}\). Subchondral cysts can frankly communicate with the joint space and are lined with fibrous connective tissue containing adipocytes and osteoblasts\(^{27}\).

The role of subchondral bone in OA appears to be complex. Although the role of the variety and sequence of subchondral bone changes in the development of OA is not yet clear, the mechanical integrity of the joint surface is disrupted and cartilage responds.

**Synovial tissue**

The synovial tissue lines in the joint cavity and consists of one or two cell layers of synovial cells. The synovial cells play a role in nourishment for the chondrocytes through the synovial fluid in the joint space. But it is also responsible for the removal of metabolites and products of matrix degradation\(^{20}\). Even though OA was traditionally seen as a non-inflammatory disease, synovitis is detectable in most patients with OA\(^{20}\). Acute inflammatory flares, characterized by redness, swelling, pain or heat are not uncommon\(^{20}\).

Serological and histological evidence of synovitis is commonly found in OA, even though OA has not been consistently associated with specific immune responses. Inflammation may contribute to the cartilage damage by producing catabolic cytokines, such as interleukin-1 (IL-1) and tumor necroses factor α (TNFα) among others, which in turn induce release of matrix metalloproteinases (MMPs), such as collagenases and stromelysines\(^{28}\). These catabolic factors are also produced by chondrocytes and are involved in normal matrix turnover as well. However, they are produced in elevated amounts in OA, contributing to joint pathology\(^{29}\). The synoval Changes in OA are minor compared to rheumatoid arthritis (RA), where aggressive invasion of synoval tissue in cartilage and bone significantly contributes to joint damage\(^{30}\). Nonetheless, there is evidence that synoval inflammation is involved in the early onset of OA and it is present in both advanced OA and in early OA when cartilage damage is less evident\(^{20}\).
Studying OA in animal models

In humans, studying the initiation and very early features of OA is very difficult. Animal models of OA can be of help for this purpose since actual biochemical, molecular, and histochemical analyses of joint tissues can be combined with longitudinal imaging and biomarker evaluation. Many OA animal models have been developed, most of them in rodents and canines but also in goat and sheep. Joint degeneration occurs spontaneously in certain breeds or can be induced chemically or mechanically. Depending on the model, the disease develops fast or slowly, in a localized, or more generalized manner, with differences in relative involvement of synovium, cartilage or bone. A frequently used OA model is the anterior cruciate ligament transection (ACLT) model in the dog. This model has also been applied in smaller animals like rats or rodents. OA is induced by transection of the anterior cruciate ligament, resulting in an unstable knee (stifle) joint and thus disruption of normal joint biomechanics. In addition to the canine ACLT model, the canine Groove model of OA has been developed. By surgically applying grooves in the cartilage of the femoral condyles, a local biomechanical and biochemical change within the joint and the cartilage itself induces generalized degeneration in the whole joint. An advantage of the Groove model over the ACLT model is the lack of instability and consequently permanent disruption of joint biomechanics, potentially allowing the (experimental OA Groove) joint to regenerate.

Advantages of experimentally induced models include the ability to define precisely the type of injury, the severity of injury, as well as the time of onset and progression (rate) of injury and to relate these events to markers of disease activity and even severity (pain)/loading by force plate analyses. Moreover, appropriate controls are available like the contralateral joint, or sham surgery groups with identical genetic background.

Risk factors studied in this thesis

Loading

*In vitro* and *in vivo* studies, including clinical (*in vivo*) studies suggest that articular cartilage chondrocyte metabolism is strongly affected by mechanical loading. While reduced loading and overloading both cause cartilage degradation, moderate levels of activity maintain normal cartilage integrity. A considerable number of studies, however, support the concept that mechanical demand that exceeds the tolerance of the joint (cartilage, bone, or ligaments) has a major role in the development and progression of OA. Physically demanding occupations, sports subjecting joints to intense loading, and obesity are associated with OA, suggesting that more intense joint loading is associated with onset and progression of joint degeneration. Unloading can be effective in reducing pain and can even result in actual cartilage repair. Examples are weight reduction, insoles, and joint distraction.

Chondrocytes’ responses to mechanical stimuli involve a number of distinct molecular pathways that link environmental signals to mechanisms that control gene expression. Injurious static compression stimulates depletion of proteoglycans and damage to the collagen network and decreases the synthesis of cartilage matrix proteins, whereas dynamic compression increases matrix synthesis activity. In response to traumatic injury, global gene expression is activated, resulting in increased expression of inflammatory mediators, cartilage-degrading proteinases, and stress response factors. Chondrocytes have receptors for responding to mechanical stimulation. Activation of these receptors can stimulate the production of matrix-degrading proteinases and inflammatory cytokines and chemokines. There is good evidence that conditions that produce increased load transfer and/or altered patterns of load distribution across the joint can accelerate the initiation and progression of OA.
Aging
The prevalence of OA rises with age. However, not all older adults develop OA and not all joints are equally affected. Although the relationship between aging and the development of OA is incompletely understood, aging changes in the musculoskeletal system contribute to the development of OA by working in conjunction with other factors such as obesity, joint injury, and genetics.

Chondrocytes from older adults exhibit many of the changes that are typical of cell senescence. Telomere shortening in chondrocytes has been reported in cells isolated from older adults. Since chondrocytes hardly divide it seems unlikely that they would experience telomere shortening due to classical replicative senescence in vivo. More likely this is due to DNA damage from reactive oxygen species. Chondrocyte senescence results in altered activity and expression of regulatory proteins that control growth and proliferation.

Cytokine and MMP production is changed in chondrocytes derived from cartilage of older adults. After stimulation with IL-1β more MMP-13 is secreted, a major mediator in type-II collagen cleavage. Furthermore, there is a decline in response to proliferative and anabolic response to growth factor stimulation. An age-related loss in the normal mitogenic response to several different growth factors has been noted. IGF-1 is well known to stimulate cartilage matrix synthesis and there is substantial evidence for an age-related decline in the ability of IGF-1 to stimulate proteoglycan and collagen production. In addition there is an age-related reduction of levels of certain growth factors in cartilage, including TGFβ.

Also oxidative damage from reactive oxygen species (ROS) is changed in aged chondrocytes. Levels of ROS increase in cartilage with aging and chondrocytes from older adults are more susceptible to ROS-mediated cell death. Finally, the number of chondrocytes decline in cartilage tissue with increasing age due to increased cell death. Increased cell death is due to less growth factor activity, the loss of survival promoting matrix proteins, and the increase in oxidative damage.

Age-related changes in the cartilage matrix could also be important in contributing to the development of OA. Articular cartilage becomes thinner with age, especially in the knee and at the patella. This is consistent with gradual loss of cartilage matrix with age as well as a decrease in cartilage hydration and cellularity. In human femoral cartilage age-related decrease in cellularity and glycosaminoglycan content was found contributing to weakening of the tissue. Age-related changes in the size, structure, and sulfation pattern of aggrecan have also been reported. Aggrecan’s abundant negatively charged sulfates, which are very hydrophilic, are responsible for maintaining the high content of water in cartilage. Aging induced changes in aggrecan constitution contribute to a loss in cartilage resiliency and hydration. There is an age-related accumulation of aggrecan fragments containing the hyaluronic acid binding region. The aggrecan fragments that remain bound to hyaluronic acid occupy the space where a newly synthesized complete aggrecan molecule would normally bind and thus result in smaller proteoglycan aggregates being present with increasing age.

Advanced Glycation Endproducts
One of the most important age-related changes in articular cartilage is the accumulation of advanced glycation end products (AGEs). AGEs are formed by reducing sugars such as glucose, fructose, or ribose, reacting with (hydroxy-)lysine or arginine in a process of non-enzymatic glycation (NEG) (figure 1). It has long been assumed that NEG is initiated solely by the spontaneous condensation of reducing sugars with free amino groups in lysine or arginine. After formation of a covalent bond between the sugar and the amino acid, subsequent reactions give rise to the formation of
AGEs\textsuperscript{66}. It is now apparent that AGE formation may also be initiated by metal-catalyzed glucose auto-oxidation and lipid peroxidation\textsuperscript{67}. Thus, there are multiple sources and mechanisms of AGE formation \textit{in vivo}. Because of the highly diverse reaction pathways leading to AGE formation, AGES with a variety of chemical structures have been identified. Some AGES are adducts to the proteins whereas others present protein-protein cross-links\textsuperscript{66,68}.

![Classical view of AGE formation](image)

**Figure 1: classical view of AGE formation\textsuperscript{67}**. Reducing sugars such as glucose or fructose react spontaneously with lysine or arginine residues in proteins. Initially a reversible Schiff base 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.

Once they are formed, AGES cannot be removed from the tissue and therefore AGES only leave a tissue when the protein involved is degraded. Since the rate of AGE accumulation is largely determined by the rate of protein turnover, accumulation of AGES in proteins occur in tissues with a low protein turnover, such as lens crystallines, skin and cartilage collagens\textsuperscript{69-72}. Cartilage has a relatively high level of pentosidine, one of the AGES, compared to other tissues\textsuperscript{73}. Detailed studies on cartilage collagen showed that all well-characterized AGES (pentosidine, $N^\alpha$-carboxymethyllysine (CML) and $N^\alpha$-carboxyethyllysin (CEL)) accumulate in cartilage collagen\textsuperscript{70,74}. Furthermore, an age-related increase of general measures of AGES (fluorescence at 370/440 nm, browning, and amino acid modification) was also observed still leaving pentosidine, the best-characterized AGE, as a suitable and highly sensitive marker of overall AGE. Although pentosidine also accumulates in aggrecan, most pentosidine (80-85\%) in aged human articular cartilage is found in collagen\textsuperscript{70,75}. This most likely results from the much slower turnover of collagen than of aggrecan\textsuperscript{76}. Consistently, the higher AGE concentration in cartilage compared with skin collagen is fully explained by the difference in turnover of collagen between the two tissues\textsuperscript{69,70,73}.
The accumulation of AGEs in collagen adversely affects the mechanical and biochemical properties of articular cartilage (figure 2).

Figure 2: AGEs increase with increasing age as well as OA. This may be due to the fact the AGEs influence the mechanical and biochemical factors of the cartilage tissue. All figures are adopted from different articles.16,77,78,16,79,2
Enhancing AGE formation *in vitro* by adding ribose or threose to cartilage explants increases the stiffness of the collagen network substantially. This increased stiffness is highly correlated with the AGE levels of collagen. Furthermore, tensile testing of articular cartilage reveals increased brittleness of the collagen network after in *vitro* enhancement of AGE cross-linking. Consistent with these results it has been shown that the stiffness of the collagen network in articular cartilage increases with age, most likely the result of AGE accumulation. The age-related increase in collagen network stiffness and brittleness as a consequence of AGE cross-linking may result in a concomitant increase in susceptibility of the collagen network to mechanically induced damage. Indeed the resistance of the collagen network in articular cartilage to fatigue damage decreases with increasing age.

AGEs also interfere with cellular processes such as adhesion of cells to the extracellular matrix, proliferation and gene expression. The age-related decrease in matrix synthesis by articular chondrocytes is largely explained by the impaired synthesis of collagen and proteoglycans at high AGE levels. Although the mechanism by which AGEs exert their effects on chondrocytes remains unclear, the presence of the receptor for AGEs (RAGE) in chondrocytes suggests a role for this pleiotropic receptor. RAGE is expressed by chondrocytes and RAGE levels in cartilage increase with both aging and the development of OA. Upon binding, RAGE triggers an intracellular signal transduction route that is only partly understood. Cell activation in response to binding of AGE-modified proteins, commonly results in upregulation of the receptor, activation of key signaling molecules such as NF-κB, and production of, for example, pro-inflammatory cytokines and reactive oxygen species.

**Advanced glycation endproducts and *in vivo* models of OA**

In combination, the increased sensitivity for mechanical damage and the decreased capacity of chondrocytes to remodel (and hence repair) their surrounding extracellular matrix render the cartilage more prone to damage, and might provide the molecular mechanism by which age-related accumulation of AGEs leads to the development of OA. Indeed, using an *in vivo* model of OA, it was demonstrated that accumulation of AGEs accelerates the progression of OA. AGE levels in the knee cartilage of beagle dogs were artificially enhanced by repeated intra-articular ribose injections. This resulted in a ~5-fold increase of pentosidine level, compared to the PBS injected joints. In this model, all joints developed OA due to surgically-induced joint instability but cartilage damage was more severe in the joints with artificially enhanced AGE levels than in the PBS injected joints. As such this study supported the role of AGEs in progression of cartilage damage. Furthermore, a comparison of collagen AGE levels in visually intact cartilage from individuals with and without focal knee cartilage degeneration (postmortem) elsewhere in the joint reveals that individuals who undergo more, or more rapid, glycation are more susceptible to developing OA.

**Thesis**

The purpose of this thesis was to further study the effects of cartilage AGE accumulation on the development of OA. As described above, AGEs accumulate with increasing age in tissues with a slow turnover such as cartilage. Age, (in)dependent of cartilage AGE accumulation, predisposes to development of cartilage degeneration as seen in OA. The tight relation between tissue AGEing and aging of an individual hampers studying these phenomena independently. In an effort to pry apart this tight relationship and to improve our understanding if AGE accumulation is one of the molecular mechanism to the development of OA, both animal *in vivo* studies and human *ex vivo* studies are described in this thesis.
**Canine in vivo studies**

Studying the effect of AGEs on cartilage damage *in vivo*, independent of age, necessitates the use of animal models. In animal models AGE levels of articular cartilage can artificially be enhanced in a short time span by supplying high amounts of highly reactive reducing sugars. This approach was previously used in the canine anterior cruciate ligament transection (ACLT) model of OA, showing more severe cartilage damage in the joints with increased AGE levels\(^8\), supporting the role of AGEs in *progression* of cartilage damage.

In this thesis the role of AGEs in the *development* of OA was studied. The ACLT model is not suitable for this purpose, since the permanent instability of the joint after surgery always induces joint degeneration. The canine Groove model of OA was previously developed, using a onetime trigger (surgically applied chondral damage) combined with temporary forced loading of the affected joint to overcome this drawback of the ACLT model. In order to apply the Groove model for the studies into the effect of AGEs on the development of OA, first further knowledge needed to be gained on the role of loading in the development of OA in this canine Groove model. The effect of loading in addition to the surgically applied chondral damage on the development of joint degeneration (OA) was studied in *chapter 2*.

Based on the results of this study, for evaluation of the effect of AGE on the development of OA, a dual approach was chosen. On the one hand, minimal surgically applied chondral damage with limited joint loading was applied; on the other hand significant mechanical stress (loading) of the joint without inducing a cartilage trauma was used. By use of minimal chondral damage and restraining joint loading it was anticipated that development of OA in normal joints would not ensue. As such in *chapter 3*, normal joints and joints with artificially enhanced cartilage AGE levels were compared after minimal chondral damage with restraining joint loading, expected to lead to OA development in the latter only.

In *chapter 4* the alternative approach was used by providing a significant mechanical stress (loading) of the joint without inducing a cartilage trauma. In case of artificially enhanced AGEing of the cartilage, OA might develop whereas in control joints, the enhanced loading will be insufficient to induce development of OA. The advantage of this approach is that the joint cartilage remains surgically untouched and as such the role of AGEs in development (not progression) of OA can be studied in the most natural form.

**Human studies**

Parallel to the animal studies, *in vivo* and *ex vivo* effects of AGEs are studied in humans. As mentioned, human *ex vivo* studies are hampered by the interference of aging with the effect of cartilage AGEing. Bypassing this effect can be done by studying a population with a small age difference but sufficiently variation in AGEing, or evaluating joint damage as detailed as possible to be able to discriminate additive effects of AGEing on the general effect of aging.

Studying the role of AGEing in the development of OA *in vivo* it is favorable to use a population with signs of early OA. The CHECK (cohort hip & cohort knee) is such a cohort, consisting of participants with knee and/or hip pain starting between 45-65 years of age\(^8\). As such the interference of age is limited, because the age of the participants is restricted. Next to this there is a focus on the knee and hip joints, often involved in OA, and representing a significant volume of the total volume of hyaline articular cartilage. This facilitated the use of systemic markers of cartilage breakdown and cartilage AGEing.

In case of studying OA no gold-standard for the evaluation of the severity of OA is available. Radiographs are most often used for this purpose. The Kellgren and Lawrence score\(^8\) is one of the most frequently used scores. However this score has several limitations because it is a rather rough combined stepwise score of 0-4, anticipating a fixed sequence of involvement of bone and cartilage changes. Digital image analysis using continuous measures of multiple variables is more precise in evaluating OA features, for example in measuring the joint space width independently of other OA related changes. For the hip such computer assisted digital
image analysis method was available, measuring the minimal and mean joint space width as a continuous variable. For the knee no adequate technique was available. Therefore, first a computer assisted model of measuring knee radiographs was developed as described in chapter 5, to evaluate knee radiographs by use of continuous measures of separate radiographic features of joint damage (KIDA; knee images digital analyses).

These two digital measures of radiographic joint damage, the conventional Kellgren and Lawrence score, and the data from the CHECK cohort allowed studying of the effects of AGEing on cartilage degeneration. In chapter 6 the skin and urine pentosidine levels of participants in the CHECK cohort are cross-sectional correlated with radiographic signs of OA to gain more information on their role in the onset of OA.

The same cohort was used to study the progression of OA over time. In chapter 7 we studied whether skin pentosidine, urine CTXII or the combination of both factors is able to predict radiographic joint damage after 5 year follow-up.

Even though detailed continuous measures of joint space narrowing were used as a measure of cartilage damage, radiographic evaluation still is a surrogate of the actual damage. Therefore, in chapter 8 the actual macroscopic, histological, and biochemical features of cartilage degeneration were assessed in a cohort of patients with end stage OA. These parameters were related to cartilage and urine AGE levels.

In chapter 9 the results of the individual studies are summarized, integrated, and discussed. A general conclusion is provided with suggestions for future studies.
References

10. Moskowitz, RW. Osteoarthritis. 2007;4ed:


Does loading influence the severity of cartilage degeneration in the Canine Groove model of OA?

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Abstract

Objective:
Many animal models are used to study osteoarthritis (OA). In these models the role of joint loading in the development of OA is not fully understood. We studied the effect of loading on the development of OA in the canine Groove-model.

Methods:
In ten female beagle dogs OA was induced in one knee according to the Groove-model. The animals were divided in groups with and without forced-loading. Forced-loading was achieved by fixing the contra-lateral limb to the trunk 3 times a week for 4 hours. After 20 weeks joint tissues of all dogs were evaluated.

Results:
Subjective evaluation revealed less movement with more loading in the forced-loading-group compared to the group without forced-loading. In both groups induction of OA resulted in macroscopical and microscopical OA changes as well as alterations in cartilage metabolism characteristic for OA. Although differences were small, for some parameters they were statistically significant more outspoken for the forced-loading-group. There were no differences between the contra-lateral healthy joints of both groups.

Conclusions:
The present study demonstrates that in the Groove-model intensified loading is not a prerequisite for the development of OA, although it adds to some extent to the severity of the OA.
Loading effect on canine groove-model of OA

**Introduction**

Osteoarthritis (OA) is considered to develop slowly over a long period of time and is often diagnosed at a relatively late stage of the disease\(^1\). The pathophysiology during onset and early non-symptomatic progression is largely unknown. For this reason, studies on early changes in the processes of human OA are important. In this respect, animal models\(^2\) may be helpful to improve insight in these early changes to improve early diagnosis and to evaluate therapies early in the course of disease.

A few years ago the canine Groove-model of OA was described\(^3-5\). In this model, surgically applied damage to the articular cartilage surface of the weight-bearing areas of only the femoral condyles, not damaging the subchondral bone and not causing joint instability, in analogy to models using cartilage defects as trigger for osteoarthritis\(^6,7\), is the trigger for joint damage. To strengthen this trigger for development of OA, loading of the affected joint is intensified by intermittently and temporarily fixing the contralateral control limb to the trunk\(^4\).

Biochemical and histological evaluation showed degenerative changes in the joint, which closely resemble those of the frequently used anterior cruciate ligament transection (ACLT) model\(^8\). Both models are considered to mimic human OA.

Evaluation of the Groove-model at 3 weeks post-surgery demonstrated a repair process, whereas at 10 weeks cartilage degeneration is evident, indicating that joint degeneration is more than simply the expression of surgically applied damage\(^5\). Evaluation at 20 and 40 weeks post-surgery demonstrated slightly progressive features of OA as seen in human OA\(^3\). It is unclear which factor is responsible for converting the repair process at 3 weeks into a process of progressive degeneration from 10 weeks on. One of the factors possibly responsible for the loss of functional repair is the applied intensified loading during the first 10 or 20 weeks.

In other animal studies there are suggestions that intensified loading adds to development of OA. Neurectomy after ligament transection in dogs accelerates the osteoarthritic features, which is expected to depend on intensified loading\(^9\). Chondral defects in non-weight bearing areas of femoral condyles in horses repair completely within 3 month in contrast to defects in the weight bearing areas (maximum follow-up 9 month), suggesting that loading contributes to development of OA after initial cartilage damage\(^10\). In transgenic Del1 mice, running activity causes an increase in incidence and severity of degenerative changes in the articular cartilage of knee joints within 15 month\(^11\). Forced mobilization of rats after ACLT and partial medial menisectomy leads to an increase in OA compared to normal mobilization, again indicating that loading is important in the development of OA\(^12\).

More importantly, also in human OA, there is an increasing body of literature suggesting that the mechanical environment of the knee during ambulation has a profound influence on the severity, progression and treatment outcome for osteoarthritis of the knee. Physically demanding occupation, sports subjecting joints to intense loading, and obesity are associated with osteoarthritis, suggesting that more intense joint loading is associated with onset and progression of joint degeneration\(^13,14\).

Based on the above described animal and human studies, intensified loading was applied, and expected essential, in the development of progressive joint degeneration in the Groove-model. However, it was never actually evaluated whether this temporary intermittent intensified loading is essential for the development of progressive degenerative features. Therefore, in the present study, the effect of intensified loading on the development of degenerative features related to OA was evaluated for the canine Groove-model.
Methods

Animals
Ten female Beagle dogs (20.5±1.8 month, 11.0±1.6 kg) were obtained from the animal laboratory of the Utrecht University, the Netherlands. They were fed a standard diet and had water ad libitum. The Institutional Animal Care and Use Committee approved the study.

Induction of OA
Surgery was carried out through a 2–2.5 cm medial incision close to the patellar ligament in the right knee. Bleeding and soft tissue damage was prevented as much as possible to prevent dominance of an inflammatory component. Ten longitudinal and diagonal grooves, depth 0.5 mm, were made in the femoral cartilage, as described before\(^4\). Damage of subchondral bone was prevented, confirmed by histology at the end of the experiment, to avoid repair activity by recruitment of bone marrow cells. Macroscopic evaluation after sacrificing of the animals showed similar groove patterns in all affected knees. The tibial plateau was left untouched. After surgery, synovium, fasciae and skin were sutured. The contralateral unoperated knee served as a control.

Loading regime
Two groups, five randomly selected animals each, were housed in indoor/outdoor pens. The animals were led out on a large patio for 4 hours/daily, starting 2 days postsurgery. Two different conditions of loading were applied. In one of both groups, the dogs were forced to load the experimental joint during 3 days/week by fixing the contralateral limb to the trunk while walking on the patio according to the original developed model (the group with intensified-loading)\(^4\). The other group was not subjected to the forced-loading but in all other aspects treated identically (group without forced-loading). Walking pattern was observed 3 times/week.

Outcome
Severity of OA was evaluated 20 weeks after surgery. After euthanizing, both hind limbs were amputated and synovium, synovial fluid, and cartilage were collected and processed under laminar flow conditions.

Synovial tissue analysis
Macroscopic synovial inflammation was evaluated on high-resolution photographs of synovium (one photograph per joint), by two blinded observers. Severity of inflammation was graded as described before\(^5\). Haematoxylin–eosin stained histological sections of three synovial tissue samples were scored by two blinded observers, by use of the slightly modified\(^15\) criteria of Goldenberg and Cohen\(^16\).

Synovial fluid MMP activity
Synovial fluid was aspirated from the joint before opening it using a syringe. The MMP activity was measured using a fluorogenic substrate TNO211-F in the presence of EDTA-free general proteinase inhibitor Complete\(^\text{TM}\). This assay is considered to represent overall MMP activity\(^17\).

Cartilage analysis
Macroscopic cartilage damage was evaluated on high-resolution photographs (one photograph for tibia, one for femur per joint) of the tibia and femur by two blinded observers as described before\(^5\).
Cartilage samples for histological and biochemical analyses were obtained from predetermined locations of the weight-bearing areas of the femoral condyles and the tibial plateau of the experimental and control joints. Locations were identically paired with the same mirrored location in the contralateral joint, representing all joint compartments (lateral, medial, femur and tibia) of each joint as described previously. Safranin-O-fast-green stained sections from four cartilage samples from the tibial plateau and four from the femoral condyles, from each knee were evaluated using the slightly modified criteria of Mankin. For biochemical analysis, cartilage samples were cultured according to standard procedures. For femoral condyles and tibial plateau, cartilage proteoglycan content, - synthesis, - retention, – release and DNA content were determined for six explants per parameter.

**DNA content**

The DNA content of the cartilage samples was determined as a measure of the cellularity of the cartilage. In part of the papain digest of the ex vivo cartilage samples DNA was stained with fluorescent dye Hoechst 33258 as described earlier. Calf thymus DNA (Sigma D-4764) was used as a reference. Auto fluorescence of cartilage is negligible.

**Proteoglycan turnover**

As a measure of proteoglycan synthesis, the rate of sulphate incorporation was determined ex vivo during 4 hours by using $^{35}$SO$_4^{2-}$ as a label. Synthesis rate is expressed as nmol of sulphate incorporated per hour per mg DNA of the cartilage (nmol/h/g). As a measure of the retention of newly synthesized proteoglycans, the release of $^{35}$SO$_4^{2-}$ labeled proteoglycans in the medium was determined, as described previously. The release of newly formed proteoglycans is expressed as percentage release of newly formed proteoglycans in 3 days (% new proteoglycan release). For the total release of proteoglycans, Alcian blue (8-GX Sigma A5268) stained and precipitated proteoglycans were quantified photometrically with chondroitin sulphate (Sigma C4384) as a reference. The total amount of glycosaminoglycans (GAGs) released is expressed as a percentage of the original tissue content (% GAG release).

As a measure of proteoglycan content of the cartilage samples, the amount of Alcian Blue stained and precipitated GAG was determined as described previously. Values were normalized to wet weight of the cartilage explants (mg/g).

**Collagen damage**

Degraded collagen was measured as described before. In short, after extraction of proteoglycans (PGs), degraded collagen molecules in the insoluble collagen network were selectively digested with α-chymotrypsin at 37°C. The supernatant, containing the fragments derived from the digested collagen molecules, was removed and hydrolyzed, as was the insoluble matrix left after α-chymotrypsin digestion. The hydroxyproline content of both pools was used to calculate the percentage of degraded collagen present in the samples.

**Calculations and statistics**

For all histochemical evaluations data from 4 cartilage explants (a minimum of 3 slides each) of each tibia and 4 explants of each femur were averaged. For biochemical evaluation data from 8 cartilage explants of each tibia and 8 from each femur were averaged. Mean values of five animals (joints) ±SD for femoral and tibial cartilage separately are presented. As this is an explorative study only an N-value of five animals per experimental group was used. Paired Student’s t-test was used to compare data of the experimental and contralateral control joints within each group. The unpaired t-test was used to analyze differences between the two groups (absolute values of the control and experimental joints separately). Two-sided p-values less than or equal to 0.05 were indicated by an asterisks (*), p-values less than or equal to
0.10 (0.05 < p ≤ 0.10) were indicated by a hash (#), representing a tendency to statistically difference, and p-values > 0.10 were considered not statistically significant (NS).

**Results**

Amount of exercise

Subjective evaluation during the 20 weeks of follow-up revealed that the dogs ‘with forced-loading’ could easily walk on three limbs with intensified full loading of the experimental joint. However, although easily possible, this appeared strenuous because of instability and the increased load, and these animals sat down frequently limiting the actual (intensified) loading time of the experimental joint. The animals ‘without forced-loading’ were much more active and the total movement time of the experimental joint was longer than the animals ‘with forced-loading’. However, because the animals can easily walk on three limbs the actual loading of the experimental joint in this group was limited, specifically in the first weeks. The animals used their fourth affected limb for stability with only partial loading (a condition quite different from that of a fully fixed limb). This resulted in significantly more movement with less loading compared to the ‘forced-loading’ group with less movement but more loading.

![Graphs showing results](image.png)

**Figure 1: Effect of forced-loading on cartilage matrix integrity**: macroscopic cartilage score (A); microscopic cartilage score (B); proteoglycan content (C); and collagen damage (D) for both femur condyles and tibia plateau of dogs with and without intensified loading. Bars represent mean ± SD of n=5 animals per group. White bars represent contralateral control knee joints, grey bars represent experimental knee joints. NS, not significant, * P ≤ 0.05, # P ≤ 0.1.
Cartilage integrity

Twenty weeks after induction of experimental OA, the affected knees of all animals of both groups clearly showed macroscopic femoral cartilage damage. No damage was found on the condylar cartilage of the control knees (figure 1A). Damage, although less pronounced, was also clear on the tibial plateau of the experimental joints compared to the control joints (figure 1A). No differences were found in the macroscopic cartilage damage between both groups.

Histology (figure 1B), demonstrated cartilage degeneration of the surgically damaged femoral compartment and the surgically untouched tibial compartment. For both, the group with and without forced-loading, the effects were statistically significant between the experimental and control knee. For the femur, but not for the tibia, the histological damage was statistically significantly more outspoken in the group with forced-loading when compared to the group without forced-loading. The individual scored items as used by the Mankin grade (structure, cells and safranine-O staining) showed each the same differences between groups (data not shown). As such, the total sum of the three items was used for statistical evaluation.

Representative micrographs for both joints of both groups of both compartments are presented in figure 2. Fibrillation of the articular surface (brackets), loss in safranine-O staining (accolades), and chondrocyte clustering (indicated by arrows) were all clearly visible, being all characteristics of osteoarthritis.

Figure 2: Representative light micrographs of cartilage obtained from joints with (right) and without (middle) intensified loading and of a contralateral control joint (left) of femoral condyles (top) and tibial plateau (bottom). Note the fibrillated surface (bracket), loss of safranine-O staining (accolade) and cell clustering (arrows) as characteristics of OA cartilage.
Proteoglycan loss (figure 1C) and collagen damage (figure 1D) were statistically significantly evident in both joint compartments in both groups when compared to the contralateral control joint (figure 1C). No differences in degree of cartilage proteoglycan content and collagen damage were found between the group with forced-loading and the one without forced-loading.

Importantly, for none of the four parameters related to cartilage integrity there was a difference (even no sign of a tendency) between the contralateral control joints of both groups, with fixation and without fixation of this joint (figure 1A-D).

**Figure 3: Effect of forced-loading on chondrocyte activity**; proteoglycan synthesis rate (A); retention of newly formed proteoglycans (B); and total proteoglycan release (C) for both femur condyles and tibial plateau of dogs with and without intensified loading. Bars represent mean ± SD of n=5 animals per group. White bars represent contralateral control knee joints, grey bars represent experimental knee joints. NS, not significant, * P≤0.05, # P≤0.01.

**PG turnover**

In the forced-loading-group proteoglycan synthesis rate (figure 3A) of femoral condyle (+110%) as well as at tibial plateau (+125%) was increased statistical significantly (p≤0.05; *) in the experimental joints compared to the contralateral control joints. In the group without forced-loading this increase was evident but statistically less pronounced (+74% and +57%, for femur and tibia, respectively; p≤0.10; #). Despite this difference in the experimental joints compared to the control joints of both groups (paired evaluation) no statistically significant
difference between the experimental joints (nor for contralateral control joints) of both groups was found (un-paired evaluation).

This for OA characteristic increased proteoglycan synthesis rate, an attempt to repair, was ineffective as the percentage release of newly formed proteoglycans (figure 3B), a measure of retention of these proteoglycans, was increased in the experimental joint compared to the contralateral control joint (+51% and +17%, for femur and tibia of the forced-loading-group, respectively and +53% and +5%, for femur and tibia of the group without forced-loading, respectively). For the tibial plateau the effect was not statistically significant for the group without forced-loading (+5%; NS) but present in the group with forced-loading (+17%; p≤0.10; #).

The total amount of proteoglycans released (resident and newly formed; figure 3C) of the experimental joints was enhanced compared to the contralateral control joints. This increased proteoglycan release was slightly more profound for the group with forced-loading (+108% and +14% for the femur and tibia, respectively) compared to the group without forced-loading (+84% and +12% for the femur and tibia, respectively). For the femoral condyles the difference between both groups had a tendency to be statistically significant (p≤0.10; #).

Importantly, for none of the parameters related to proteoglycan turnover (chondrocyte activity) there was a statistically significant difference between the contralateral control joints of both groups, with fixation and without fixation of this joint (figure 3A-C).

Figure 4: Effect of forced-loading on synovial inflammation; macroscopic score (A); microscopic score (B); and MMP activity (C) of dogs with and without intensified loading. Bars represent mean ± SD of n=5 animals per group. White bars represent contralateral control knee joints, grey bars represent experimental knee joints. NS, not significant, * P≤0.05, # P≤0.01.

Synovial inflammation
Macroscopic evaluation of the synovial tissue showed mild inflammation in the groups with and without forced-loading of the experimental joint compared to the contralateral control joint (figure 4A). Microscopic evaluation corroborated these results and showed for both groups similar results (figure 4B), just a mild inflammatory response. No differences were found between the group with and without forced-loading, neither for the experimental joints nor for the contralateral control joints.
MMP activity in de synovial fluid (figure 4C) demonstrated increased activity in both groups compared to the control group. No statistically significant difference was found between the two experimental conditions.

Discussion
This study compared features of osteoarthritis due to surgically applied chondral damage combined with and without transient intermittent forced-loading of the affected joint. Parameters of cartilage integrity, chondrocyte activity, and synovial inflammation were evaluated. It appeared that differences between both study groups were hardly present; only chondrocyte activity in general and the macroscopic cartilage score, for the femoral condyles only, demonstrated slightly more severe changes in favor of forced-loading.

This finding contrasted our original idea and hypothesis, based on literature and clinical findings, that loading is important in development and progression of OA. However, evaluation of the actual loading and mobility of the animals demonstrated a major drawback in the study set-up. Although initially it was intended to create two groups of animals, one with significant loading of the surgically affected joint and one with minimal loading of the affected joint, this was not unambiguously achieved. As this drawback was not anticipated, we made only subjective and descriptive observations of the intensity and frequency of the loading patterns of the animals. The actual difference was that the animals ‘without forced-loading’ had more movement with less loading compared to the ‘forced-loading’ group with less movement but more loading. As such, the loading per time unit may have been comparable. Additionally, more movement in the group without forced-loading may have led to more shear stresses on the cartilage tissue, stresses that may be pathological because of the incongruence of the articular surfaces due to the surgically applied damage. This is a drawback of the set-up of the study, on the other hand is a representation of daily practice in exploiting the Groove-model and maybe even a representation of daily clinical practice. Not only actual loading but also mild loading with more and better movement may accelerate development and progression of features of OA. In this respect pain relief in clinical practice should be accompanied by regimens to limit unnecessary damage.

It appeared that in both groups features of OA developed. This was demonstrated by multiple parameters for the surgically damaged femoral condyles as well as for the surgically untouched tibial plateau. Of major importance was the observation that the contralateral control joints did not differ between both groups. This study for the first time very clearly demonstrated that fixing the contralateral joint to the trunk of the animal, 3 days per week for 4 hours a day, had no effect on any of the parameters of the control joints measured; not even a tendency towards changes in the control joint due to the forced-loading of the contralateral joint was found.

However, despite the absence of huge differences between both groups, minor, in some cases statistically significant differences in degree of degenerative features between both groups were observed. This supports the assumption that intensified loading contributes to development of degenerative changes in the Groove-model of OA. Interestingly, the parameters of chondrocyte activity, more than cartilage integrity demonstrated these differences. This fits well with the idea that the changes in chondrocyte activity indicate very early changes that take a longer time to result in actual changes in cartilage integrity.

In literature concerning human OA, increasing evidence is available that loading and unloading of the joint has major impact on the course of osteoarthritis. Several factors might be involved in the role of loading/unloading in the course of OA. It might be directly related to chondrocyte activity precipitating the different loading patterns, or it might be the extra cellular matrix that is vulnerable to overload. In this respect the accumulation of advanced glycation endproducts (AGEs) with increasing age, resulting from
the spontaneous reaction of reducing sugars with proteins, has been reported to lead to increased susceptibility of cartilage to mechanical damage\textsuperscript{26,27}. Irrespective of the contribution of (over)loading to the development and progression of OA, the present study demonstrates that in the described model, intensified loading is not a prerequisite to the development of OA, although it adds in a limited way to the severity of cartilage degeneration. Apparently, movement with mild loading leads to almost similar effects as intensified full loading which restricts movement.

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References


Elevation of cartilage AGEs does not accelerate initiation of canine experimental osteoarthritis upon mild surgical damage.

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Abstract

Objectives: Osteoarthritis is a highly prevalent disease, age being the main risk factor. The age-related accumulation of advanced-glycation-endproducts (AGEs) adversely affects the mechanical and biochemical properties of cartilage. The hypothesis that accumulation of cartilage AGEs in combination with surgically induced damage predisposes to the development of osteoarthritis was tested in vivo in a canine model.

Methods: To artificially increase cartilage AGEs, right knee joints of 8 dogs were repeatedly injected with ribose/threose (AGEd-joints). Left joints with vehicle alone served as control. Subsequently minimal surgically-applied cartilage damage was induced and loading restrained as much as possible. Thirty weeks after surgery, joint tissues of all dogs were analyzed for biochemical and histological features of OA.

Results: Cartilage pentosidine levels were ~5 fold enhanced (p=0.001 vs. control-joints). On average, no statistically significant differences in joint degeneration were found between AGEd and control-joints. Enhanced cartilage pentosidine levels did correlate with less cartilage proteoglycan release (R=-0.762 and R=-0.810 for total and newly-formed proteoglycans, respectively; P = 0.028 and 0.015 for both).

Conclusions: The current data support the diminished cartilage turnover, but only a tendency towards enhanced cartilage damage in AGEd articular cartilage was observed. As such, elevated AGEs do not unambiguously accelerate the development of early canine OA upon minimal surgical damage.
Introduction

Osteoarthritis (OA), with a high prevalence and increasing incidence due to the aging population, having a large impact on the patient’s quality of life, is characterized by progressive cartilage damage, bone changes, and secondary synovial inflammation. As yet, the pathogenesis of OA is largely unknown. Several factors have been reported to predispose to the development of OA, such as genetic background, overweight, joint laxity and muscle weakness. However, undisputedly, the most important risk factor for development of OA is age. The incidence of OA increases strongly with age: >50% of the population over 60 years of age is affected. However, there are still many uncertainties how age contributes to the onset and progression of OA. Age-related changes in the articular cartilage are suggested to play an important role in the susceptibility of cartilage to OA.

One of the major age-related changes in articular cartilage is the spontaneous modification of proteins by non-enzymatic glycation resulting in the accumulation of advanced glycation endproducts (AGEs). Non-enzymatic glycation is a posttranslational modification of proteins by reducing sugars. The spontaneous condensation of reducing sugars with free amino groups in lysine or arginine residues on proteins leads to the formation of advanced glycation endproducts (AGEs). Pentosidine, a fluorescent AGE formed between lysine and arginine residues, is frequently used as marker for AGEs. AGEs are formed in all proteins, and can only be removed from the tissue when the protein is removed. The low turnover of proteins in articular cartilage results in an abundant accumulation of AGEs in this tissue with increasing age.

AGEs are known to affect physical and chemical properties of proteins. Tissue strength is dependent upon the amount of crosslinks present and accumulation of AGEs is correlated with increased tissue stiffness of cartilage. Moreover, an increase in AGEs makes the cartilage more brittle, making the tissue more prone to mechanical damage. In addition to mechanical changes AGEs also interfere with cellular processes. It has been demonstrated that increased AGE levels lead to decreased proteoglycan turnover (synthesis and release) of articular cartilage. Altogether, these data suggest that increasing levels of AGEs in articular cartilage results in an abundant accumulation of AGEs in this tissue with increasing age.

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Studying the effect of AGEs on cartilage damage in vivo, independent of age, necessitates animal models in which AGE levels of articular cartilage can artificially be enhanced by supplying high amounts of reducing sugars. This method was used in the canine anterior cruciate ligament transection (ACLT) model of OA. AGE levels in the knee cartilage of beagle dogs were artificially enhanced ~ 5-fold by repeated intra-articular ribose injections. In this model, all joints developed OA due to joint instability but cartilage damage was more severe in the joints with artificially enhanced AGE levels than in the PBS-injected joints. As such this study supported the role of AGEs in progression of cartilage damage.

In the present study the role of AGEs in the development (initiation) of OA was studied. For this purpose minimal surgically-applied chondral damage was applied. By use of minimal chondral damage and restraining joint loading it was anticipated that OA in normal joints would not develop spontaneously. Joints with artificially enhanced cartilage AGE levels were hypothesized to be more sensitive to the OA-inducing trigger and were expected to develop OA.
glycation and surgical induced OA

Methods

**Animals:** Thirteen female Beagle dogs (age 16.4 ± 3.5 months and weight 9.5 ± 1.7 kg) were obtained from the animal laboratory of the Utrecht University, the Netherlands. They were fed a standard diet and had water *ad libitum.*

**Enhancement of AGE levels:** After one week of acclimatization, the right knee of eight animals was injected with a combination of 300 mM ribose and 5 mM threose in PBS (total volume of 2 ml; twice weekly for 7 weeks; further referred to as ‘AGEd-joint’). The left contra-lateral knee was injected at the same moment with same volumes of PBS (further referred to as ‘PBS-injected joints’). All intra-articular injections were performed under a short sedation (Dormitor®/AntiSedan® Pfizer Animal Health). After the last intra-articular injection, dogs were allowed to recuperate for 2 weeks.

**Induction of OA:** Subsequently, the femoral cartilage of both joints was surgically damaged according to procedures used for the canine Groove model, but less severe\(^\text{15-18}\). Surgery was carried out through a 2–2.5 cm medial incision close to the patellar ligament of the knee. Bleeding and soft tissue damage was prevented as much as possible to avoid dominance of a surgically-induced inflammatory component. In utmost flexion, 4 grooves (instead of normally at least 10 in the classical Groove model) were applied on the weight-bearing parts of the femoral condyles without damaging the subchondral bone. The tibial plateau was left untouched. The animals were permanently housed individually in indoor pens (3.5m\(^2\) per animal) to keep movement (and with that loading) of the joints to a minimum. To put in perspective, normally the animals are led out on a patio of 200m\(^2\) for 4 hours per day in large groups. Cartilage pentosidine levels (as a measure of AGE) and severity of joint degeneration were evaluated 30 weeks after surgery.

For negative and positive controls, in the five additional dogs (not artificially AGEd), OA was induced according to the classical Groove model in the right joint using a minimum of 10 grooves and forced loading of the affected joint by fixing the control joint (further untreated) to the trunk of the animal for a few hours a day, 3 days a week. Only biochemical features of joint degeneration were evaluated 20 weeks after induction\(^\text{15-18}\).

The Utrecht University Medical Ethical Committee for animal studies approved the study.

**Outcome:** After euthanizing, both hind legs were amputated immediately *post mortem.* High resolution photographs were taken of cartilage and synovial tissue for macroscopic evaluation. Subsequently synovial tissue and cartilage of both joints were collected and processed within 2 hours. Procedures were carried out under laminar flow conditions.

**Pentosidine levels:** Cartilage pentosidine levels as a measure of AGE accumulation were measured as described previously\(^\text{19}\). In short: at least three randomly taken cartilage samples of each joint were pooled and reduced overnight, sequentially treated with L-Cysteine (5mM, Sigma), EDTA (50 mM, Sigma), NaOH (1 M, Merck) and papain (3% v/v. Sigma). Collagen pentosidine content and amino acid composition were determined by high-performance liquid chromatography (HPLC) according to standard procedures\(^\text{20,21}\). The pentosidine content of collagen samples is expressed as millimoles per mole collagen, assuming 300 hydroxyproline residues per triple-helical collagen molecule.

**Macroscopic cartilage damage and synovial tissue inflammation** were evaluated on digital high-resolution photographs, by two observers unaware of the source of the photographs, using criteria as described previously\(^\text{17}\). Severity of cartilage damage was graded from 0-4: 1=smooth surface, 2=roughened, 3=slightly fibrillated, 4=fibrillated. The scores of the two observers were averaged for the tibial joint surface (maximum score 4) and synovial tissue (maximum score 6); average values subsequently used for statistical evaluation.

**Histological cartilage damage and synovial tissue inflammation** were evaluated using samples from the weight bearing tibial plateau, and from the infra-patellar synovium, from predefined locations as described before\(^\text{15}\). Fixed samples were sectioned and stained with safranin-O-
fast-green-iron-hematoxylin and hematoxylin-eosin for cartilage and synovium, respectively. Sections were scored blinded and in random order by two independent observers using the OARSI score for cartilage (max.=36) and synovium (max.=18)\textsuperscript{22}. The scores were averaged for the specimens from the tibial surface (n=4, each) and synovial tissue (n=3) and for the two observers; average values of each joint were subsequently used for statistical evaluation.

**Chondrocyte activity:** Cartilage samples of the tibial plateau were cultured according to standard procedures\textsuperscript{17}. Cartilage proteoglycan content, -synthesis, -retention of newly formed proteoglycans, and -release were determined for eight explants per parameter of fixed weight bearing locations with identical locations at the contralateral joint\textsuperscript{17}. All samples were handled individually. The average result of the eight samples was taken as representative of that tibial joint surface and was used for statistical analysis\textsuperscript{17}.

**PG content:** To measure the PG content of the cartilage samples, the amount of tissue glycosaminoglycans (GAGs) was determined. The GAGs in the papain digest of cartilage samples were precipitated, stained with Alcian Blue, and quantified photometrically by the change in absorbance at 620 nm with chondroitin sulphate as reference. Values were expressed per wet weight of the cartilage tissue (mg/g).

**Proteoglycan turnover:** As a measure of proteoglycan (PG)-synthesis, the rate of sulphate incorporation was determined ex vivo. After one hour of pre-culture, 370 kBq Na\textsubscript{2}\textsuperscript{35}SO\textsubscript{4} in 10 µl DMEM was added to each sample. After 4 hours labeling, the cartilage samples were washed three times with medium for 45 minutes. Subsequently, samples were cultured for three days without label. Cultures were stopped by washing two times with cold (phosphates buffered saline (PBS) and freezing of the samples. GAGs in a papain digest of cartilage samples were precipitated with Alcian Blue and \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2–}-labeled GAGs were measured by liquid scintillation counting. The total sulphate incorporation rate of each cartilage sample was calculated using specific activity of the medium and was normalized for labeling time and wet weight of the explants (nmol/h.g).

**Release of newly formed proteoglycans.** To determine the release of newly synthesized PGs as a measure of the retention of these PGs, the release of \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2–}-labeled GAGs in three day culture medium was measured. GAGs were precipitated from the medium with Alcian Blue, as described\textsuperscript{17}. The \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2–}-labeled GAGs were measured by liquid scintillation counting and the release was calculated using the specific activity of the medium normalized to the wet weight of the explants. The release of newly formed PGs is corrected for the synthesis rate and expressed as percentage release of newly formed PGs in three days (% new PG release).

**PG release.** For the total release (loss) of PGs, GAGs in the culture medium were precipitated and stained with Alcian Blue and quantified as described above. The total amount of GAGs released is expressed as a percentage of the PG-content (% GAG release).

**Calculations and statistics:** For each animal a single value was obtained for each parameter by averaging the multiple analyses. In total 8 animals were used and as such this study might be considered explorative. Importantly, for cartilage parameters only the surgically untouched tibial plateau cartilage was analyzed to prevent interference of the surgical damage, which was applied to the femoral cartilage only. These values of each animal were averaged and mean values of eight (or 5 for the positive and negative control) animals ± SD are presented.

To analyze differences between the PBS-injected and AGEd-joints a non-parametric paired Wilcoxon test was used. To analyze differences with the (positive and negative) control joints the non-parametric non paired Mann-Whitney U test was used. For comparison of differences in chondrocyte activity parameters with differences in pentosidine levels spearman correlation was used; all 2-sided P values \( \leq 0.05 \) considered statistically significant.


**Results**

*Pentosidine levels:* Intra-articular injections with ribose/threose resulted in enhanced pentosidine levels in each individual dog. On average there was a 5-fold higher cartilage pentosidine level in the AGEd-joints compared to PBS-injected joints (p<0.001, Fig. 1 solid line), with a clear variation between dogs.

![Figure 1: Cartilage pentosidine levels of the joints.](image)

*Cartilage integrity:* Thirty weeks after induction of experimental OA, the affected knees of all animals showed minimal macroscopic cartilage damage (Fig. 2A, light and dark grey bars; score of 1 of a max score of 4). Fig. 2B shows a representative micrograph of cartilage from the surgically untouched tibial plateau of the PBS (top) injected and AGEd (bottom) joints. Fig. 2C shows representative histology of the tibial plateau of the PBS (top) and AGEd (bottom) joints. The average microscopic OARSI score is shown in Fig. 2D (light and dark grey bars). Also microscopy showed a minimal joint degeneration, OARSI score of 3-5 of a max score of 36. No difference between the macroscopic and microscopic scores of the AGEd and the PBS-injected joints were found (Fig. 2A and 2D, respectively). Both macroscopy and histology of the cartilage tissue was compared with data of the classical Groove model (white and black bars). For both scores the outcome lies in between the control and experimental joints.
Figure 2: Cartilage integrity. A: Average macroscopic cartilage score, B: Representative photographs of tibial surface obtained from PBS-injected (top) and AGEd (bottom) joints of tibial plateau, C: Representative micrographs of cartilage histology obtained from PBS-injected (top) and AGEd (bottom) joints of tibial plateau. Note the mild fibrillated surface, mild loss of safranine-O staining and minimal cell clustering as characteristics of very mild degree of joint degeneration, and D: microscopic cartilage score, of tibial cartilage from PBS-injected and AGEd-joints. Bars represent mean ± SD of n=8 animals per group. Data of the present experiment are presented in the context of a negative control (left white bar; Groove model) and experimentally induced OA as appositive control (right and black bar; Groove model; n=5). Light grey and dark grey bars in the middle represent the PBS-injected and AGEd-joints of the present experiment, respectively (n=8). P values between the PBS-injected and the AGEd-joints are indicated, considering P≤0.05 as statistically significant different.

Synovial inflammation: The macroscopically judged synovial inflammation in the PBS-injected and the AGEd-joints was on average low to moderate (Fig. 3A, (light and dark grey bars); score of 2 of a max score of 5), indicating that synovial inflammation was minimal, slightly enhanced expectedly due to the repeated intra articular injections. Also the microscopic histological score of the synovium was low to moderate (Fig. 3D, (light and dark grey bars); score of 4-5 of a max score of 18). Representative micrographs are given in Fig. 3B for the macroscopy and in Fig. 3C for histology. On average no differences in macroscopic and microscopic score (Fig. 3A and 3D, respectively) were found for the AGEd and the PBS-injected joints. Both macroscopy and histology of the synovial tissue was compared with data of the classical Groove model (white and black bars). For both scores the outcome lies in between the control and experimental joints, although for the macroscopic score this is less evident.
Figure 3: Synovial inflammation. A: Average macroscopic cartilage score B: Representative photographs of synovial tissue obtained from PBS-injected (top) and AGEd (bottom) joints. Note the mild synovial inflammation C: Representative micrographs of synovium histology obtained from PBS-injected (top) and AGEd (bottom) joints of tibial plateau, and D: microscopic cartilage score. Bars represent mean ± SD of n=8 animals per group. Data of the present experiment are presented in the context of a negative control (left white bar; Groove model) and experimentally induced OA as appositive control (right and black bar; Groove model; n=5). Light grey and dark grey bars in the middle represent the PBS-injected and AGEd-joints of the present experiment, respectively (n=8). P values between the PBS-injected and the AGEd-joints are indicated, considering P≤0.05 as statistically significant different.

Cartilage biochemistry and chondrocyte activity: As was seen for the cartilage integrity and the synovial tissue inflammation also proteoglycan (PG) content, -synthesis, newly formed -release, and total -release were not statistically significant different for the PBS-injected and the AGEd-joints (Fig. 4, light and dark gray bars in the middle of each set of four bars). When the biochemical data are compared to control and OA joints from the classic Groove model (Fig. 4 the white bars; left, representing unoperated control joints and Fig. 4 the black bars; right, representing the OA joints) it is clear that the severity of cartilage damage in the present study is positioned in-between normal healthy and early OA joints. In fact it also demonstrates that the AGEd-joints are in general in between the PBS-injected joints and the early OA joints. The PBS-injected joints are in-between the normal healthy joints and the AGEd-joints. Assuming a stepwise increase in severity of damage there was a correlation between severity of damage and the different conditions from healthy control joints, via PBS-injected joints with minimally surgically damage, AGEd-joints with minimal surgical damage, to experimentally induced OA joints (correlation coefficients above 0.8, p values <0.001 except for PG synthesis (p=0.20), for all biochemical parameters; Fig. 4). Similarly this was also seen for histology (fig 2D and 3D).
Figure 4: Biochemical markers of cartilage matrix integrity and chondrocyte activity of PBS-injected and AGEd-joints. A: total proteoglycan content, B: proteoglycan synthesis, C: % release of newly formed proteoglycans (measure of retention of newly formed proteoglycans), and D: % total proteoglycan release. Data of the present experiment are presented in the context of a negative control (left white bar; Groove model) and experimentally induced OA as appositive control (right and black bar; Groove model; n=5). Light grey and dark grey bars in the middle represent the PBS-injected and AGEd-joints of the present experiment, respectively (n=8). Bars represent mean ± SD. Correlation coefficients for the stepwise change in biochemical parameters (control, PBS-injected, AGEd, and OA joints) are shown.

Relationship with pentosidine: The significant variation in pentosidine levels in the AGEd-joints, allowed us to analyze possible relationships between the pentosidine levels and the outcome parameters. The difference between the pentosidine levels of the AGEd and PBS-injected joints of each animal is correlated with the percentage change of cartilage parameters between AGEd and PBS-injected joints of the same animal. For the release of total and newly formed PGs a strong negative relationship was found with the enhanced pentosidine levels between AGEd and PBS-injected joints (R= -0.762, p=0.028 and R= -0.810, p=0.015, respectively; Fig. 5A and B). For proteoglycan synthesis, no statistically significant relationship with pentosidine levels was found, although the tendency was negative as well (high pentosidine correlated with low synthesis and vice versa; R=0.389, data not shown)
glycation and surgical induced OA

Figure 5: Correlation between the differences in cartilage pentosidine between PBS-injected and AGEd-joints and percentage change between both contra-lateral joints, for cartilage newly formed (A) and total (B) proteoglycan release. Proteoglycan release is negatively correlated with the change in pentosidine content. Correlation coefficients (R) and p values are indicated, considering P≤0.05 as statistically significant different.

Discussion
The present study could not demonstrate a clear difference in cartilage damage in AGEd-cartilage compared to control cartilage, upon minimal surgically applied damage. Compared to the positive control (clear OA features of the original Groove model) and negative control (healthy cartilage), artificially aging of the cartilage appeared to accelerate development of cartilage damage (OA), not reaching statistical significance. Additionally, high cartilage AGE levels demonstrated a low cartilage proteoglycan release, corroborating a diminished turnover of proteoglycans due to the AGEing of the tissue.

In this study the pentosidine levels in the AGEd-joints were approximately five-fold increased compared to the PBS-injected joints. PBS-injected joints had AGE levels expected for dogs of this age\[14\]. Only once before, artificial enhancement of AGEs was used in vivo in a canine model; also demonstrating a 5-fold increase in cartilage AGE levels\[14\]. It has been demonstrated that cartilage pentosidine in humans from young (about 20 years) to old (about 80 years) also increased 5 fold\[12\]. This means that the outcome of these experiments can be translated into the human situation so leading to more information about the development of OA and with that potential therapeutic strategies. As such it is concluded that the artificial aging was successful and of relevance to human conditions.

In the present study minor changes, characteristic of joint degeneration, were found in the PBS-injected joints, as compared to the negative controls (no treatment). The triggers for induction of joint degeneration were purposely kept to a minimum. The surgically-induced cartilage damage was restricted to a maximum of 4 grooves on femoral condyles only, whereas in the regularly performed Groove model OA at least 10 grooves are made \[18\]. Additionally, loading of the joints was minimized, knowing that loading adds to development of OA\[23\]. In the present study the animals were permanently housed individually in indoor pens (3.5 m\(^2\)), the minimal reasonably acceptable space, to keep movement and with that loading of the joints to a minimum. This in clear contrast to active exercise in large groups on a large patio in case of the classical Groove model.Apparently, the minimal surgically
applied damage of the femoral condyles and the remaining minimal loading is sufficient for minimal development of joint damage in the PBS-injected tibial joints surfaces. It should be kept in mind that the time for development of joint degeneration in the classic Groove model is 20 weeks\textsuperscript{15} whereas it was 30 weeks for the AGEd animals in the present study. This prolonged follow-up was specifically chosen to provide an increased time window to enhance the chance for differences in the development of joint damage between the PBS-injected and AGEd-joints.

Most interesting, the minimal condylar surgical-induced damage and minimal joint loading did not result in spontaneous healing but actually resulted in development of damage on the untouched tibial plateau. Studies on the spontaneous healing or progression of damage of articular cartilage defects have been performed in several animal models, including dogs\textsuperscript{17,24,25}. Clearly even the minimal joint damage of the femoral condyles in the present study, with minimal loading is not healed in a period of 30 weeks but even slightly progressed to the tibial plateau.

However, the artificial aging of the cartilage only marginally accelerates the cartilage degeneration. No significant differences were observed although compared to the control group a tendency towards more damage was seen at a biochemical level, although less than in the classic Groove model without enhanced cartilage AGEing. It might well be that this minor difference was not related to development (initiation) of joint degeneration due to the artificial aging, but that it was due to slight progression of the degeneration already started. This corroborates the previous canine study where artificial AGEing was able to accelerate progression of OA study upon ACLT\textsuperscript{14}. As such in the present study the original approach to study development of OA was hampered by the minor development of joint degeneration in the control PBS-injected joints.

Interestingly, it appeared that within the group of AGEd-joints, proteoglycan release (turnover) was the lowest in those joints with the highest cartilage AGE levels. This corroborates previous findings that increased AGE levels in cartilage lead to increased cross-linking of proteins and with that to diminished release of these macromolecules\textsuperscript{10}. Also degradation of AGE-modified collagen by matrix metalloproteinases is impaired compared to unmodified collagen\textsuperscript{26}. This will impair repair activity of the cartilage. Despite this phenomenon, also observed in the artificially AGEd-joints in this study, changes were insufficient to clearly drive the development of joint degeneration in the present study set-up. In conclusion, despite the fact that enhanced cross-linking of macromolecules by the AGEs restrains loss of proteoglycans, corroborating the diminished turnover of old cartilage, and a tendency toward enhanced cartilage damage in the artificially AGEd-joints, the present data do not support a predominant role for enhanced cartilage AGE levels in development of joint degeneration in early OA. As such the clinical relevance of AGEing of cartilage in development of OA remains obscure.

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References


Artificial ageing of canine knee cartilage by elevation of cartilage AGEs does not induce cartilage damage upon enforced joint loading in vivo.

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Abstract
Objectives: Osteoarthritis is a highly prevalent disease, for which age and loading are important risk factors. Age-related accumulation of advanced glycation endproducts (AGEs) adversely affects the mechanical and biochemical properties of cartilage, and may - in combination with enforced loading - induce osteoarthritis in a canine in vivo model.

Methods: To artificially increase cartilage AGEs, right knee joints of 8 dogs were repeatedly injected with a mixture of ribose/threose (AGEd-joints); left knee joints injected with PBS alone served as a control. The dogs were exercised actively by housing them in outdoor pens and additionally daily exercise on a patio in large groups was achieved to enforce loading. After almost 2 years joint tissues of all dogs were analyzed for biochemical and histological features of OA.

Results: Cartilage pentosidine levels were 20 fold enhanced (p=0.001 vs. control-joints) at the end of the experiment. Irrespectively, on average no clear differences in joint degeneration were found between joints with and without enhanced pentosidine levels except for reduced proteoglycan synthesis in the AGEd-joints (p=0.05).

Conclusions: Significant artificial AGEing of a joint, leading to cartilage mimicking a pentosidine level of very old animals, is insufficient to develop OA following prolonged active joint loading.
**Introduction**

Osteoarthritis (OA) is a highly prevalent degenerative joint disease, involving the whole joint. The disease progresses slowly and develops over many years. Several risk factors have been identified, of which the most important are loading and aging.

Several animal studies show that intensified loading can lead to OA\(^1\). In the canine Groove model of OA forced loading by fixing the limb to the trunk results in increased severity of OA\(^5\). For human OA, there is an increasing body of literature suggesting that mechanical loading can lead to OA. More intense joint loading is associated with onset and progression of joint degeneration\(^6\).

In addition to loading, age has been identified as an important risk factor for OA. One of the major age-related risk factors is the accumulation of advanced glycation end products (AGEs), a non-enzymatic reaction between proteins and sugars\(^7\). During aging accumulation of AGE in articular cartilage occurs, due to the slow turnover of the cartilage matrix proteins\(^7\). These AGEs cause increased stiffness and brittleness of the cartilage tissue\(^8,9\) and diminish repair capacity\(^10\), making the cartilage tissue more prone to mechanical damage. As such the age-dependent AGE accumulation does not cause OA by itself, but it is assumed to make the joint more prone to development and progression of OA. The identification of AGE accumulation as a molecular mechanism contributing and/or predisposing to the development of OA opens new avenues for OA treatment. On different stages in AGE formation therapeutic strategies are possible, but also breakers of AGE are therapeutic possibilities\(^11\).

In the past the effect of AGEing on progression of OA in vivo was tested by artificial enhancement of AGEs in cartilage using repeated intra-articular injections of ribose. Subsequently joint instability was induced by anterior cruciate ligament transection (ACLT). In the ribose-injected joints features of OA was more severe than in the control (PBS-injected) joints\(^12\). This study focused on the role of AGEs in the progression of OA.

In a parallel performed canine in vivo study, artificially enhanced cartilage AGEs impaired loss of proteoglycans, corroborating the diminished turnover of proteoglycans in old cartilage. Moreover, marginally increased cartilage damage in these artificially AGEd-joints was observed after a minor surgically induced cartilage trauma causing minor joint degeneration by itself\(^13\). However, also in this study, the effect observed was on progression of the (minor) damage already existing.

The present approach was to clarify the role of AGEs in the development of OA. It is hypothesised that following artificially significantly enhanced AGEing of knee joint cartilage, the cartilage resilience would be impaired (increased brittleness and diminished repair capacity) to such an extent that prolonged enforced loading (in the absence of any surgically induced ligament or cartilage trauma) would lead to OA development. In control joints, this enforced loading is expected to be insufficient to induce development of OA. The advantage of this approach is that the joint cartilage remains surgically untouched and as such the role of AGEs in solely the development (and not progression) of OA can be studied.
Methods

Animals: 8 female Beagle skeletally mature dogs (age 13.1±1.4 month and weight 8.9±1.6 kg) were obtained from the animal laboratory of the Utrecht University, the Netherlands. They were fed a standard diet and had water ad libitum. They were housed in indoor/outdoor facilities (mean surface 4.2m²) with free entrance to the outdoor pens. A significant step-up from outside to inside and a spring-forced closing door between outside and inside, enforce mechanical demands on the stifle joints. Most importantly, five days a week for four hours a day for almost 2 years the dogs were exercised on a large patio (mean surface 141m²) as a group allowing free movement with frequent attendance of a biotechnician stimulating exercise. The Utrecht University Medical Ethical Committee for animal studies approved the study.

Enhancement of AGE-levels: After one week of acclimatization, the right knee of all animals was injected with a combination of 300 mM ribose and 5 mM threose in PBS (2ml; twice weekly; 7 weeks). Based on previous experiments this scheme leads to on average a 5 fold increase in cartilage pentosidine as a measure of cartilage AGE. These injections were repeated according to the same scheme after a year to further increase cartilage AGE levels (in week 62-69; ‘AGEd-joints’). The left contra-lateral knees were injected with same volumes of PBS (at both injection series; ‘PBS-injected-joints’). All intra-articular injections were performed under a short sedation (Dormitor®/AntiSedan® Pfizer Animal Health).

Outcome: After almost 2 years (week 92) the animals were euthanized to evaluate the joints for actual cartilage AGE levels and characteristics of OA. After euthanizing, both hind legs were amputated immediately post-mortem. High-resolution photographs were taken of cartilage and synovial tissue for macroscopic evaluation. Subsequently synovial tissue and cartilage of both joints were collected and processed within 2 hours. Procedures were carried out under laminar flow conditions.

Pentosidine levels, as a measure of AGE accumulation in cartilage, was measured in at least 3 randomly taken cartilage samples of each joint. The samples were pooled and tissue pentosidine levels were determined by reversed-phase-high-performance-liquid-chromatography after acid hydrolysis as described previously.

Macroscopic cartilage damage and synovial tissue inflammation were evaluated on digital high-resolution photographs, by two observers unaware of the source of the photographs using criteria as described previously. Severity of cartilage damage was graded from 0-4: 1=smooth surface, 2=roughened, 3=slightly fibrillated, 4=fibrillated. The scores of the two observers were averaged for each joint surface (tibia and femur, maximum score 4 each) and synovial tissue (maximum score 6); average values were used for statistical evaluation.

Histological cartilage damage and synovial tissue inflammation were evaluated using four samples from the weight bearing tibial plateau and femoral condyles each, and three from the infra-patellar synovium, from predefined locations. Fixed samples were sectioned and stained with safranin-O-fast-green-iron-hematoxylin and hematoxylin-eosin for cartilage and synovium, respectively. Sections were scored blinded and in random order by one observer using the OARSI score for cartilage (maximum score 36) and synovium (maximum score 18). The scores were averaged of the specimens from each joint surface (n=4, tibia and femur each) and synovial tissue (n=3); average values were subsequently used for statistical evaluation.
**PG content:** To measure the PG content of the cartilage samples, the amount of tissue GAGs was determined. The GAGs in the papain digest of cartilage samples were precipitated, stained with Alcian Blue, and quantified photometrically by the change in absorbance at 620 nm with chondroitin sulphate as reference. Values were expressed per wet weight of the cartilage tissue (mg/g).

**Proteoglycan turnover:** As a measure of proteoglycan (PG)-synthesis, the rate of sulphate incorporation was determined *ex vivo*. After one hour of pre-culture, 370 kBq Na$_2^{35}$SO$_4^{2-}$ in 10 ul DMEM was added to each sample. After 4 hours labeling the cartilage samples were washed three times with medium for 45 minutes. Subsequently, samples were cultured for three days without label. Cultures were stopped by washing two times with cold (phosphates buffered saline (PBS) and freezing of the samples.

Glycosaminoglycans (GAGs) in a papain digest of cartilage samples were precipitated with Alcian Blue and $^{35}$SO$_4^{2-}$-labeled GAGs were measured by liquid scintillation counting. The total sulphate incorporation rate of each cartilage sample was calculated using specific activity of the medium and was normalized for labeling time and wet weight of the explants (nmol/h.g).

To determine the release of newly synthesized PGs as a measure of the retention of these PGs, the release of $^{35}$SO$_4^{2-}$-labeled GAGs in three day culture medium was measured. GAGs were precipitated from the medium with Alcian Blue, as described. The $^{35}$SO$_4^{2-}$-labeled GAGs were measured by liquid scintillation counting and the release was calculated using the specific activity of the pulse medium normalized to the wet weight of the explants. The release of newly formed PGs is corrected for the synthesis rate and expressed as percentage release of newly formed PGs in three days (% new PG release)

For the total release (loss) of PGs, GAGs in the culture medium were precipitated and stained with Alcian Blue and quantified as described above. The total amount of GAGs released is expressed as a percentage of the PG-content (% GAG release).

**Calculations and statistics:** For each animal for each parameter a single value was obtained for each parameter by averaging the multiple analyses. In total 8 animals were used. As no differences for tibial and femoral cartilage were observed the average of both compartments was taken for all parameters. These values of each animal were averaged and mean values of eight animals ± SD are presented. To analyze differences between the PBS-injected and the AGEd-joints a non-parametric paired Wilcoxon test was used. Tests were 2 sided and P values less or equal to 0.05 were considered significant.
Results

Pentosidine levels
As expected, PBS-injected joints had low normal AGE-levels for dogs of this age\(^{12}\). The double ribose/threose injection series resulted in on average 20-fold increase in pentosidine level. The increase in AGEs was statistically significant from the PBS-injected joints (Fig. 1). Note that after the first series of injections already a 5-fold increase is reached (cf. data in\(^{13}\)).

![Cartilage pentosidine levels of the joints.](image)

Figure 1: Cartilage pentosidine levels of the joints. Each line represents the difference in pentosidine levels for the PBS-injected joints (left) and AGEd-joints (right). The solid line represents the change in mean value (n=8) between PBS and AGEd-joints. The amount of pentosidine is statistically significant (p<0.001) higher in the AGEd compared to the PBS-injected joints.

Cartilage integrity
Almost 2 years after the start of the experiment the affected knees of all animals showed minimal macroscopic cartilage damage (Fig. 2A; score of 0.33 of a maximum score of 4). On average, there was a minor increase in the macroscopic score of the AGEd compared to the PBS-injected joints (p=0.084). Also microscopic cartilage damage was limited (Fig. 2B; score of 1.2 of a maximum score of 36). On average, there was no difference between the AGEd and PBS-injected joints.

Synovial inflammation
The synovial inflammation as judged macroscopically and microscopically was minimal in the AGEd as well as in the PBS-injected joints (Fig. 2C and D, respectively). There were no differences between the PBS-injected and AGEd-joints.

Chondrocyte activity: Changes in chondrocyte activity with respect to proteoglycan turnover precedes the actual changes in the cartilage matrix, therefore chondrocyte activity was determined as well. The PG content and the release of total as well as newly formed PGs showed no difference between the PBS-injected and the AGEd-joints (Fig. 3A, C, and D, respectively). For the PG synthesis a statistically significant lower activity was found for the AGEd compared to the PBS-injected joints (p=0.05, Fig. 3B).
Cartilage glycation and enforced loading

Figure 2: Cartilage matrix integrity in PBS-injected (white) and AGEd-joints (grey): macroscopic cartilage score (A); microscopic cartilage score (B); synovial inflammation (C); and microscopic synovium score (D). Bars represent mean ± SD of n=8 animals per group. P values between the PBS-injected and the AGEd-joints are indicated, considering P≤0.05 as statistically significant different.

Figure 3: Chondrocyte activity in PBS-injected (white) and AGEd-joints (gray): PG content (A); proteoglycan synthesis rate (B); retention of newly formed proteoglycans (C); and total proteoglycan release (D). Bars represent mean ± SD of n=8 animals per group. P values between the PBS-injected and the AGEd-joints are indicated, considering P≤0.05 as statistically significant different.

Discussion

Artificial significant aging of knee joint cartilage by increasing cartilage AGE-levels did not result in development of features of osteoarthritis in two years actively exercised dogs. Previously it has been shown that artificially aging of knee joint cartilage by increasing cartilage AGE-levels result in enhanced progression of features of osteoarthritis in the canine ACLT model. In a more recent study, artificially aging of knee joint cartilage by increasing cartilage AGE-levels 5-fold resulted upon minimal surgically induced damage in a tendency to enhanced joint degeneration. In the present study AGE-levels up to 20-fold were reached upon a second series of injections. In humans AGE-levels increase over a life time between the age of 20 and 80 between 3 and 33-fold. Absolute AGE-levels however, are much higher in humans than in dogs. Although translation to the human situation remains difficult, the
absence of an observed effect in OA development is not expected to be the result of insufficient glycation of the cartilage of the experimental joints. In this study it was expected that artificially enhancing AGE-levels in combination with active loading over a prolonged period of time would result in initiation (development) of joint degeneration as compared to joints without enhanced AGE-levels. However, this was not observed. The only observed difference was that proteoglycan synthesis was lower in the AGE-ed compared to the PBS-injected joints. This is consistent with earlier observations that AGEing of cartilage results in diminished proteoglycan turnover. In vitro studies show that higher pentosidine levels strongly correlated with a decrease in proteoglycan synthesis rate, suggesting that glycation of the extracellular matrix (ECM) affects chondrocyte activity. This was not due to a decrease in cell number with age, but strongly correlated with the amount of cartilage pentosidine levels in the cartilage. Similar observation were done in a previous in vivo study demonstrating high cartilage AGE-levels correlating with low cartilage proteoglycan release (turnover). The decreased proteoglycan synthesis may indicate impaired repair capacity, which in the long run (apparently beyond the time-span of our current study) may contribute to OA development. It should be noted that once OA starts to develop, increased synthesis of proteoglycans is observed, likely as an apparent attempt to repair the damaged tissue.

Mechanical loading and follow-up after artificial AGEing might still have been insufficient to demonstrate that AGES indeed predispose to development of OA. Several studies showed that a mechanical demand that exceeds the tolerance of the joint has a major role in the development and progression of osteoarthritis. Repetitive increased contact stress on articular cartilage surface can initiate or accelerate joint degeneration. AGE accumulation results in increased tissue stiffness and brittleness. AGES by themselves do not cause OA, they are anticipated to predispose to the development of the disease. A trigger, most likely mechanical in nature, is needed to induce the disease process. In other words, AGE accumulation makes the joint more prone to develop disease. In the present study it was intended to exceed the tolerance of loading in specifically the artificially AGE-ed joints to initiate cartilage damage. Based on the absence of degenerative features it might be questioned whether the mechanical loading was sufficient. Whether a real insult/trauma is needed to induce actual damage and whether the threshold for damage is lower in artificially AGE-ed compared to control joints remains unsolved. A previous study with a minor surgically induced cartilage trauma and minimal joint loading was also unable to clearly induce joint degeneration in artificially AGE-ed-joints when compared to PBS-injected joints. The minor effects observed in that study were suggested to be due to progression of joint degeneration instead of induction because control joints did already have signs, although minimal, of cartilage damage. This fits the canine study where artificial AGEing was able to clearly accelerate progression of ACLT induced OA.

In conclusion, artificial AGEing of a joint, leading to cartilage mimicking a pentosidine level of very old animals, is in the current study insufficient to develop OA in case of prolonged active joint loading.

Acknowledgements
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References


Age-related decrease in proteoglycan synthesis of human articular chondrocytes: the role of 
Knee Images Digital Analyses (KIDA): a novel method to quantify individual radiographic features of knee osteoarthritis in detail.

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Abstract

Objective
Radiography is still the golden standard for imaging features of osteoarthritis (OA), such as joint space narrowing, subchondral sclerosis, and osteophyte formation. Objective assessment, however, remains difficult. The goal of the present study was to evaluate a novel digital method to analyse standard knee radiographs.

Methods
Standardized radiographs of 20 healthy and 55 OA knees were taken in general practice according to the semi-flexed method by Buckland-Wright. Joint space width (JSW), osteophyte area, subchondral bone density, joint angle, and tibial eminence height were measured as continuous variables using the newly developed Knee Images Digital Analysis (KIDA) software on a standard PC.

Two observers evaluated the radiographs twice, each on two different occasions. The observers were blinded to the source of the radiographs and to their previous measurements. Statistical analysis to compare measurements within and between observers was performed according to Bland and Altman. Correlations between KIDA data and Kellgren & Lawrence (K&L) grade were calculated and data of healthy knees were compared to those of OA knees.

Results
Intra- and inter-observer variations for measurement of JSW, subchondral bone density, osteophytes, tibial eminence, and joint angle were small. Significant correlations were found between KIDA parameters and K&L grade. Furthermore, significant differences were found between healthy and OA knees.

Conclusion
In addition to JSW measurement, objective evaluation of osteophyte formation and subchondral bone density is possible on standard radiographs. The measured differences between OA and healthy individuals suggest that KIDA allows detection of changes in time, although sensitivity to change has to be demonstrated in long-term follow-up studies.
Introduction

Osteoarthritis (OA) is a slowly developing degenerative joint disease, characterised by pain and functional disability. Structural changes, such as damage of the articular cartilage, changes in the subchondral bone, and secondary inflammation, are expected to originate at least in part these clinical symptoms. Despite all efforts in research on OA over the past years, a clear definition of the disorder and proper diagnostic criteria remain difficult to identify. One of the main reasons for this difficulty is the (apparent) inconsistency between radiographic OA and symptomatic OA. There is hardly a correlation between radiographic scores (representing structural changes) and clinical symptoms. In fact in clinical practice radiographs are primarily used to exclude other underlying reasons of pain and functional disability. Even in the case of surgical intervention, clinical symptoms, more than radiographs, are directive in decision-making. Indeed, the way radiographic images are presently read and scored makes it difficult to detect subtle changes in a short time span. It is generally appreciated that significant changes in radiographic scores take at least 1 year or even 2 years.

Both the limited association of the presently available radiographic scores with clinical symptoms and the limited discriminating abilities in case of disease progression or changes in progression due to treatment, tempted many to study novel imaging techniques, the most obvious being Magnetic Resonance Imaging (MRI). However, radiography continues to be the golden standard in imaging of OA joints since the technique is cheap, fast, and available in all hospitals. The Food and Drug Administration (FDA) (guidance for industry at www.fda.gov/cder/guidance) still demands radiographic changes to prove disease-modifying efficacy of treatment strategies. Moreover, the Group for the Respect of Ethics and Excellence in Science (GREES) recommended joint space narrowing on radiographs, in addition to pain and function as a co-primary endpoint to determine the efficacy of disease-modifying drugs. Reliable objective quantitative analysis of radiographs is difficult. Except for Joint Space Width (JSW) narrowing, parameters such as subchondral sclerosis and osteophyte formation are mostly integrated in overall grading systems that comprise multiple OA related changes on radiographs, e.g. the most frequently used composite score of Kellgren & Lawrence (K&L). This makes such grading systems less sensitive to small changes in individual parameters. Although also grading systems for individual features are used, both types of grading systems use very rough stepwise scoring (ordinal variables) instead of gradual detailed changes (continuous variables). This all will add to the limited correlation between radiographic changes and clinical symptoms and to the limited sensitivity to change. To improve the sensitivity to change in evaluation of radiographs, quantification of individual features of OA in continuous variables are required. Up to now, only joint space width can be given as a continuous variable. On weight bearing radiographs, the distance between the bone ends (i.e., JSW) corresponds (at least to a certain extent) with thickness of the articular cartilage. Objective measurement of the radiographic joint space width has been reported for the hip, the ankle, and the knee. Recently, quantitative measurements of joint space narrowing have been described to be more sensitive to change than semiquantitative ratings. The accuracy of measurements of joint space width can be improved by digital image analysis of the radiographs, by standardisation of radiography of the joint, and by correction for radiographic magnification.

At present, objective quantitative evaluation of radiographs is mainly limited to measurement of minimal and mean JSW. Objective measurement of radiographic subchondral sclerosis, osteophytes, tibial eminence, and the angle of the knee joint has not yet been developed.
Therefore, we have developed and evaluated a novel method to quantify in detail a broad spectrum of individual radiographic features of knee OA: Knee Images Digital Analysis (KIDA).

**Patients and Methods**

*RADIOGRAPHY*

Semi-flexed (metatarsophalangeal [MTP]) Posterior Anterior (PA) radiographs of the tibia-femoral joint were taken under full weight bearing according to the protocol of Buckland-Wright\(^{23,24}\). The standard settings were 55kV, 5 mA s, and Focal Film Distance was 1.0 m with the knee against the detector. Radiographs were taken with an aluminium step wedge on the lateral side of the knee, against the detector (film) within the field of exposure, in order to quantify bone density changes in time and correct for possible magnification of the radiograph. As in routine clinical practice, different technicians took the radiographs. The images of 20 healthy knees (4 male/ 16 female; age 30.8 ± 1.5 years, range 22–43 years) and of 55 knees with OA features (OA according to the ACR criteria\(^{25}\); 31 male/ 24 female; age 54.5 ± 1.5 years, range 30–82 years) in different stages of the disease were used for evaluation of OA related features using KIDA. This implicates that the study does not validate standardisation of radiographic procedures (as has been done before\(^{23,24}\)) but only the digital analyses.

*KIDA*

KIDA is a software application for interactive analysis of radiographs of the knee, based on ImageXplorer, developed at the Image Sciences Institute, Utrecht, the Netherlands. Only radiographs that have been taken according the defined procedures can be analysed. To facilitate standard evaluation, images are presented on the screen with the fibula located on the left side of the image. Enlargements and contrast adaptations on screen can be performed, when ever required; they do not influence the final outcome. Six consecutive steps are performed as follows:

1. **Identification of the step wedge reference:** The aluminium step wedge reference (15 cm x 3 cm; thickness varies from 1.2 cm to 4 cm in 15 steps) is included in the protocol (see figure 1) in order to be able to derive a measure for bone density and correct for magnification of the radiograph. The observer interactively indicates the four corners of the wedge, which results in the outline of the wedge, automatically drawn by the program. The application calculates the size of the image pixels using the indicated length of the step wedge (compared to the known length of 15 cm). Additionally, based on the indicated outline of the wedge the computer identifies, with safe margins, the different steps of the wedge (see figure 1). The program calculates the maximum grey value in the region of correct exposure in the characteristic curve of the X-ray film or detector. In case pixels have a value above the maximum reliable value they are given this maximum value for further calculations. To show the observer whether the maximum value is reached these pixels are coloured green on the screen. In such cases there is an underestimation of the actual density.
Figure 1: Presentation of KIDA. Lines and circles are interactively indicated as described in Patients and Methods. KIDA provides data on JSW, subchondral bone density, osteophyte area, the height of the tibial eminence, and the angle of the joint. The inset shows the analysis of osteophytes, minimum JSW, and the angle of the joint.

2. Identification of the joint: A framework of 4 lines (L1, L2, L3, L4), which can separately be repositioned by the observer, is initially placed by the program (see figure 1). L1 is a line touching the lateral bone edges of femur and tibia excluding osteophytes (lateral side of the joint), L2 is a line touching the two points of greatest curvature of the femoral condyles, L3 is a line touching the two lowest points within the floor of the tibial plateau, L4 is a line touching the medial bone edges of femur and tibia excluding osteophytes (medial side of the joint). When both the anterior margin and the posterior margin of the tibial plateau are visible, the anterior margin is used.

3. Defining the bone-cartilage interface and subchondral area: Subsequently, the program calculates the position of 4 perpendiculars upwards on line L2 in the lateral compartment and 4 in the medial compartment, with the same procedure downwards on line L3. The 4 perpendiculars are placed at a mutual distance 1/20 of A-B and C-D, respectively. The outer perpendiculars are placed at 2/15 A-B from A and B (lateral and medial compartment, respectively), and 2/15 C-D from C and D for the tibia (white lines in figure 1). Points A to D are the intersections of L2 and L3 with lines L1 and L4 (see figure 1). Simultaneously, circles (with a diameter of 1/20 A-B or C-D) are automatically placed with its centres on the perpendiculars. The location of the perpendiculars and the size of the circles were chosen to cover the major part of the area of interest.
The operator then interactively indicates the intersection of the perpendiculars with the edges of the femur (the cartilage bone interface) by positioning the bottom (for femur) or top (for tibia) of the 16 circles manually at the bone cartilage interface (see Figure 1). The program calculates the distance between each pair of circles as a measure of joint space width (4 locations in the lateral compartment and 4 in the medial compartment). In addition, the program calculates the mean intensity of the radiograph in each circle. Comparison of the intensity in the circles with the intensity of the step wedge reference (with known thicknesses; by local interpolation analysis) gives a measure of bone density in mm aluminium equivalent.

4. **Defining the top of the tibial eminence:** The program places two circles, which need separately to be repositioned by the observer to indicate the top of the tibial eminence. The observer positions the bottom of the circle at the top of the tibial eminence. The program calculates the distance from the bottom of both circles to the line L3 as a measure for the height of the tibial eminence.

5. **Defining the osteophyte margins:** Four circles at each of the compartments of the joint (diameter = 3/20 CD and 1/10 CD for femur and tibia, respectively) are placed by the program (figure 1). The size of these circles is based on the average natural curve of the bone edges of the human tibia and femur. The observer needs to reposition these circles to place them exactly into each corner of the bones forming the joint, following the original lines of the bone. Subsequently, the observer indicates the border of the osteophyte by clicking at multiple points on the outer osteophyte margin. Only the osteophyte margin within a quadrant is indicated. Although this leads to an underestimation of the actual area, it adds to reproducibility of the procedure. The program calculates for each of the four compartments the osteophyte area that is defined by the manually indicated osteophyte outer margin and the boundary of the circle positioned by the observer.

6. **Joint angle and minimum joint space width:** From the intersection points that determine the bone cartilage interface (see under step 3), the two central circles of the four circles in the medial and lateral compartment each are used for regression analysis. This is performed for the femur and tibia providing two lines (see inset of Figure 1) that define the joint angle as calculated by the program. This procedure is more accurate than taking the angle between L2 and L3 because it uses for each line four instead of two points. A negative angle indicates joint space narrowing on the medial side of the joint. Subsequently, the program gives within the joint edges a vertical line at the narrowest point between these regression lines, suggesting the minimum joint space width. Because of the curved and not fully congruent bone margins the observer may need in a second step to reposition the two horizontal lines (this does not influence the calculation of the angle) and the vertical line in order to indicate the actual minimum joint space. The program calculates the distance between the intersection points with the horizontal lines as a measure of minimum joint space width.

**Extraction of the quantitative results**

The entire interactive procedure takes less than 10 minutes per radiograph and provides the following data. As measures for joint space width, the distance between each pair of circles on femur and tibia (4 values for the lateral side and 4 values for the medial side), the mean distance for each compartment (lateral and medial) of the joint, and the mean distance for the whole joint are given in mm. As measures for subchondral bone density the mean density of each of the 16 subchondral bone circles, the mean of the four circles for each of the four compartments (lateral femur, medial femur, lateral tibia, and medial tibia), and the mean value of all circles are given in mm aluminium equivalents. The height of the tibial eminences are given in mm. The osteophyte area of each of the four outer osteophyte regions is given in...
The angle of the joint is given in degrees and the minimum joint space width is given in mm.

**METHODS OF EVALUATION**

Two observers evaluated the radiographs using KIDA, each on two different occasions with an interval of at least one week: one biomedical scientist (AM) and one fully unrelated non-academic. The observers were blinded to the source of the radiographs and to their previous measurements. Statistical analysis to compare measurements within and between observers was performed according to Bland and Altman. In brief, the intra-observer variation in the digital analysis (KIDA) was determined by plotting the difference in the first and the second score against the mean of these two observations. Inter-observer variation in the digital analysis (KIDA) was determined by plotting the difference in the second score from one observer and the second score from the other observer against the mean of the two scores. The distance between the mean of measurement differences (the solid line in the Bland and Altman plots) and zero indicates the bias. For intra- and inter-observer variation 95% confidence intervals of the differences were calculated. Assuming no systematic bias (mean of differences equals 0), 1.96 times the standard deviation (SD) defines the smallest detectable difference (SDD). This evaluation of observer reproducibility is distinctly different from a test-retest reproducibility where in addition to observer reproducibility the process of taking the images is integrated in the evaluation. To compare KIDA parameters with the most frequently used grading system for OA, radiographs were also scored using the K&L grade (PV). Individual KIDA data were compared to the overall K&L grade. Additionally, KIDA data of healthy knees were compared to those of OA knees.

**STATISTICAL ANALYSIS**

The Mann-Whitney U test was used to analyse differences between the values measured between healthy and OA knees. Spearman correlation coefficients of the individual KIDA data and the K&L grade data were calculated. P<0.05 was considered to be statistically significant.

**Results**

KIDA can be learned in less than an hour to a non-academic. The duration of a full evaluation including data storage is between 5 and 10 minutes per radiograph.

**INTRA- AND INTEROBSERVER VARIATION IN MEASUREMENT OF KIDA PARAMETERS**

Table 1 depicts for each parameter mean actual values ± SD for all radiographs and intra- (A1-A2) and inter- (A2-B2) observer variation in the measurements. With respect to the latter, the mean difference between first and second score (Mean Δ), SD, range, and 95% C.I. of differences are given.

As an example, figure 2(A) and (B) shows the differences between two measurements of observer A (A1-A2) plotted against the mean of these measurements in the evaluation of minimum joint space width (A) and subchondral bone density of the medial femur (B). Similarly, figure 2(C) and (D) shows the inter-observer variation of the same parameters by plotting the differences between the second measurements of observer A and B (A2-B2) against the mean of these measurements. The solid horizontal line depicts the mean of the differences, while the dashed horizontal lines depict the mean ± 1.96 times the SD of the differences.
Table 1: Intra- and inter-observer variations according to Bland and Altman. Mean ± SD depicts the mean actual value of each parameter of all radiographs. Mean Δ = mean difference between first and second observation of all radiographs; SD = standard deviation of mean differences between first and second observation; range = range of differences between first and second observation; 95% C.I. = mean difference ± 1.96 times the SD.

<table>
<thead>
<tr>
<th>Observer</th>
<th>Mean ±SD</th>
<th>Mean Δ</th>
<th>SD</th>
<th>Range</th>
<th>95% C.I.</th>
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<td>Mean JSW measurements (mm)</td>
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<tr>
<td>A1-A2</td>
<td>5.1 ±1.2</td>
<td>0.04</td>
<td>0.44</td>
<td>-2.58 – 1.06</td>
<td>-0.82; 0.90</td>
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<tr>
<td>A2-B2</td>
<td>5.1 ±1.1</td>
<td>-0.05</td>
<td>0.52</td>
<td>-2.48 – 2.03</td>
<td>-1.10; 0.96</td>
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<td>Lateral JSW measurements (mm)</td>
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<td>A1-A2</td>
<td>6.1 ±1.5</td>
<td>0.03</td>
<td>0.78</td>
<td>-5.02 – 2.05</td>
<td>-1.50; 1.56</td>
</tr>
<tr>
<td>A2-B2</td>
<td>6.1 ±1.5</td>
<td>-0.04</td>
<td>0.99</td>
<td>-5.10 – 4.38</td>
<td>-1.98; 1.90</td>
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<td>Minimum JSW measurements (mm)</td>
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<td>4.2 ±1.6</td>
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<td>0.34</td>
<td>-1.41 – 1.66</td>
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<tr>
<td>A2-B2</td>
<td>4.2 ±1.6</td>
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<td>0.38</td>
<td>-1.83 – 0.49</td>
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<tr>
<td>A1-A2</td>
<td>28.6 ±4.6</td>
<td>0.00</td>
<td>0.55</td>
<td>-1.81 – 2.25</td>
<td>-1.08; 1.08</td>
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<tr>
<td>A2-B2</td>
<td>28.6 ±4.6</td>
<td>-0.11</td>
<td>0.48</td>
<td>-1.29 – 1.96</td>
<td>-1.05; 0.83</td>
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<td>Subchondral bone density medial femur (mm Alu Eq.)</td>
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<tr>
<td>A1-A2</td>
<td>29.6 ±4.2</td>
<td>-0.04</td>
<td>0.54</td>
<td>-1.66 – 2</td>
<td>-1.10; 1.02</td>
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<td>A2-B2</td>
<td>29.7 ±4.2</td>
<td>-0.17</td>
<td>0.60</td>
<td>-2.23 – 2.21</td>
<td>-1.35; 1.01</td>
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<tr>
<td>Subchondral bone density lateral femur (mm Alu Eq.)</td>
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<tr>
<td>A1-A2</td>
<td>31.3 ±4.6</td>
<td>0.06</td>
<td>0.43</td>
<td>-1.36 – 2</td>
<td>-0.78; 0.90</td>
</tr>
<tr>
<td>A2-B2</td>
<td>31.3 ±4.6</td>
<td>-0.09</td>
<td>0.56</td>
<td>-1.39 – 3.29</td>
<td>-1.19; 1.01</td>
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<td>Tibial eminence lateral (mm)</td>
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<tr>
<td>A1-A2</td>
<td>10.0 ±2.1</td>
<td>-0.07</td>
<td>1.26</td>
<td>-6.04 – 4.38</td>
<td>-2.54; 2.40</td>
</tr>
<tr>
<td>A2-B2</td>
<td>10.0 ±2.1</td>
<td>-0.03</td>
<td>0.90</td>
<td>-2.97 – 3.34</td>
<td>-1.79; 1.73</td>
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<tr>
<td>Tibial eminence medial (mm)</td>
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<tr>
<td>A1-A2</td>
<td>11.6 ±2.0</td>
<td>-0.08</td>
<td>0.98</td>
<td>-5.28 – 2.22</td>
<td>-2.00; 1.84</td>
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<tr>
<td>A2-B2</td>
<td>11.7 ±1.8</td>
<td>-0.22</td>
<td>1.44</td>
<td>-10.23 – 3.58</td>
<td>-3.04; 2.60</td>
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<td>Osteophyte lateral tibia (mm²)</td>
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<tr>
<td>A1-A2</td>
<td>6.4 ±6.5</td>
<td>-1.34</td>
<td>4.11</td>
<td>-22.71 – 7.66</td>
<td>-9.40; 6.72</td>
</tr>
<tr>
<td>A2-B2</td>
<td>6.7 ±7.4</td>
<td>0.53</td>
<td>3.43</td>
<td>-10.82 – 11.34</td>
<td>-6.19; 7.25</td>
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<tr>
<td>Osteophyte medial tibia (mm²)</td>
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<tr>
<td>A1-A2</td>
<td>9.9 ±6.8</td>
<td>-0.31</td>
<td>2.36</td>
<td>-9.97 – 5.37</td>
<td>-4.94; 4.32</td>
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<tr>
<td>A2-B2</td>
<td>9.3 ±6.4</td>
<td>1.23</td>
<td>2.97</td>
<td>-5.37 – 13.39</td>
<td>-4.59; 7.05</td>
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<tr>
<td>Osteophyte lateral femur (mm²)</td>
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<td></td>
<td></td>
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<tr>
<td>A1-A2</td>
<td>5.4 ±5.8</td>
<td>-0.71</td>
<td>3.46</td>
<td>-11.55 – 19.05</td>
<td>-7.49; 6.07</td>
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<tr>
<td>A2-B2</td>
<td>5.3 ±6.6</td>
<td>0.95</td>
<td>5.36</td>
<td>-29.51 – 13.02</td>
<td>-9.56; 11.46</td>
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<td>Osteophyte medial femur (mm²)</td>
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<tr>
<td>A1-A2</td>
<td>3.7 ±7.3</td>
<td>-0.11</td>
<td>1.64</td>
<td>-4.95 – 4.39</td>
<td>-3.32; 3.10</td>
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<tr>
<td>A2-B2</td>
<td>3.6 ±7.2</td>
<td>0.55</td>
<td>3.99</td>
<td>-20.96 – 9.27</td>
<td>-7.27; 8.37</td>
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<td>Angle between femur and tibia (degrees)</td>
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<tr>
<td>A1-A2</td>
<td>3.0 ±2.0</td>
<td>-0.11</td>
<td>1.03</td>
<td>-5.45 – 2.69</td>
<td>-2.13; 1.92</td>
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<tr>
<td>A2-B2</td>
<td>3.0 ±2.1</td>
<td>-0.08</td>
<td>1.10</td>
<td>-4.41 – 2.99</td>
<td>-2.24; 2.09</td>
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</table>

Knee Images Digital Analysis (KIDA)
In almost all cases, the inter-observer variation was larger than the intra-observer variation (see the 95% C.I. in table 1), although no huge differences were observed. The differences in measurement of all parameters did not relate to the actual value of the parameter: a large and a small JSW both showed similar differences in measurements between two observations (see representatives in figure 2).

Figure 2: Representative Bland and Altman plots for intra- and inter-observer variations of individual parameters of KIDA. The mean difference between two observations is depicted (solid horizontal lines) with 1.96 times SD of the measured differences (dashed lines). A+B: differences between two measurements of observer A (A1-A2) are plotted against the mean of these measurements in the evaluation of minimum joint space width (A) and subchondral bone density of the medial femur (B). C+D: differences between the second measurements of observer A and B (A2-B2) are plotted against the mean of these measurements.

JSW
For observer A there were small differences between the first and the second measurements for mean, lateral, medial, and minimum JSW (see table 1; compare ‘Mean Δ’ with its ‘SD’ with the actual mean values ±SD). As an example the intra-observer variation in measurement of minimum JSW is given in figure 2A. Intra- and inter-observer variations (see table 1) in measurement of medial and minimum JSW were slightly smaller than those in measurement...
of mean and lateral JSW. No systematic bias was found in the JSW measurements (small distance between ‘Mean $\Delta$’ and zero). Assuming one observer (A), the SDD (1.96 times the SD) for mean, lateral, medial, and minimum JSW was 0.86 mm, 1.53 mm, 0.67 mm, and 0.49 mm, respectively, in a range from 0 mm (no JSW left) to 9.7 mm (the maximum JSW width measured).

**SUBCHONDRAL BONE DENSITY**
For observer A, there were small differences between the first and the second subchondral bone density measurements of lateral tibia, medial tibia, lateral femur, and medial femur (see table 1; compare ‘Mean $\Delta$’ with its ‘SD’ with the actual mean values ±SD). The inter-observer variation was similar to the intra-observer variation (table 1 and figure 2D compared to 2B). The differences in subchondral bone density measurements did not appear to be related to the actual value of the measure for subchondral bone density (table 1 and figure 2B and D). No systematic bias was found in the subchondral bone density measurements. Assuming one observer (A), the SDD for lateral tibia, medial tibia, lateral femur, and medial femur were 1.06 mm Alu Eq., 0.84 mm Alu Eq., 1.08 mm Alu Eq., and 0.84 mm Alu Eq., respectively in a range from 18.2 (the smallest measure of bone density, which was assessed at the medial femur side) to 36.0 mm Alu Eq (the highest measure of bone density).

**EMINENCE**
There were also small differences between the first and second observation of observer A in measurement of the lateral and medial eminence (see table 1). Again no systematic bias was found in the eminence measurements. The SDD for lateral and medial eminence were 2.47 mm and 1.92 mm, respectively in a range from 4.1 mm (the minimal height, which was measured at the lateral side) to 16.2 mm (the maximum height).

**OSTEOPHYTES**
Differences between the first and second observation of observer A in osteophyte measurement were relatively large, compared to the other parameters (see table 1). A small systematic bias was found in osteophyte measurement within one observer as well as between two observers (a ‘Mean $\Delta$’ range up to 1.34). The SDD for lateral tibia, medial tibia, lateral femur, and medial femur were 8.1 mm$^2$, 4.6 mm$^2$, 6.8 mm$^2$, 3.2 mm$^2$, respectively in a range from 0 mm$^2$ (the minimal area) to 35.0 mm$^2$ (the maximum area).

**ANGLE BETWEEN FEMUR AND TIBIA**
Differences between the first and second observation of observer A in measurement of the angle of the joint were also small (see table 1). The SDD was 2.0$^\circ$ in a range from 0$^\circ$ to 9.1$^\circ$. For these calculations the angle was defined as an absolute value (viz. negative angles as a result of medial JSW narrowing were taken as a positive value). In fact, 90.7 % of the angles were negative (medial JSW narrowing) and 9.3 % were positive (lateral JSW narrowing). On average, the angle for medial JSW narrowing was $-3.1^\circ \pm 2.1$ and for lateral JSW narrowing $+2.7^\circ \pm 2.4$ (mean ±SD).
Figure 3: Comparison of individual parameters of KIDA with the overall K&L grade for OA. Values of individual radiographs with the median values for each K&L grade (horizontal line) are depicted. N = 30, 23, 12, and 10 for K&L grade 0, 1, 2, and 3, respectively.

Correlation between Individual KIDA Parameters
Most of the individual KIDA parameters correlated statistically significantly with other KIDA parameters. Strikingly, minimum JSW correlated statistically significantly with almost all other individual KIDA parameters, whereas the other JSW parameters (mean, lateral, medial) hardly correlated statistically with the other parameters (Table 2). It was also interesting to see that subchondral bone density in the tibia correlated well with the bone density in the femur and that also lateral and medial bone density correlated well despite uni-compartmental JSW narrowing in most cases.
COMPARISON WITH K&L GRADE
Individual KIDA parameters correlated statistically significantly with the overall K&L grade as is shown in Figure 3 and table 2, except for measurement of tibial eminence and joint angle deviation. As expected, there was a good correlation between osteophyte measurement and K&L grade (R = 0.57). But interestingly this was also found for e.g. bone density parameters. Importantly, for each of the parameters within one K&L grade a large variation exists in KIDA grading. For example, within a K&L gradation of 0 a large range in minimum JSW and osteophyte measurement is present.
Table 2: Correlations between individual KIDA parameters and K&L grade. Spearman correlation coefficients are given. Asterisks indicate statistical significant correlation coefficients, while the non-significant values are given in grey. High significant correlation coefficients (≥ 0.5) are given in bold font.

<table>
<thead>
<tr>
<th></th>
<th>Subchondral bone density</th>
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<th>Tibial eminence</th>
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<th>K&amp;L</th>
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<tr>
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<td>femur medial femur</td>
<td>femur medial tibia</td>
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</table>

66
DIFFERENCES BETWEEN HEALTHY AND OA KNEES

Differences between healthy and OA knees can be objectively measured using KIDA. Statistical significant differences were observed for all KIDA parameters except for the lateral JSW (see figure 4) and the joint angle deviation (2.6 ±0.4° vs 3.2 ±0.3° for healthy and osteoarthritis knees, respectively). K&L grade differed also statistically significantly between healthy and OA knees (K&L = 0.3 ±0.1 and 1.3 ±0.1, respectively).

Discussion

In this study we have described a novel digital method for evaluation of radiographs of OA knees: KIDA. We have compared KIDA to the frequently used K&L grading system. In addition, we used the new method to evaluate differences in KIDA parameters in healthy and OA knees. The study did not evaluate radiographic procedures (test-retest reproducibility) but focussed on the reproducibility of KIDA. This limits the conclusions that can be drawn in regard to calculations for the number of patients to be included for clinical trials because variation in the radiographic procedures (even though we used a validated standard23,24) is expectedly greater than that in the KIDA measurement. Test-retest evaluations will be performed in the near future.

The results demonstrate KIDA to be a reliable method to quantify and document (for follow-up) the individual radiographic parameters of knee OA. The small inter-observer variations in KIDA measurements indicate that similar results will be obtained when different observers evaluate knee radiographs. To our knowledge, KIDA is the first method that provides quantification of subchondral bone density, osteophyte area, and the height of the tibial eminence in addition to joint space width measurement and measurement of the joint angle on standard knee radiographs (all as a continuous variable). The statistical significant differences between healthy and osteoarthritis knees demonstrate the usefulness of the KIDA parameters. Relative mild OA joints were included as indicated by an average K&L grade of 1.3 which is the most relevant group for evaluation of follow-up, e.g. in case of treatment. The observed SDD suggest sufficient distinguishing capacity for all parameters, although longitudinal follow-up has to prove this.

The large variation in individual KIDA parameters within a single K&L grade indicates the power of KIDA parameters to distinguish a gradual change in joint damage for individual parameters. This clearly makes KIDA evaluation more sensitive than grading according to K&L. Many other methods of radiography and computer evaluation are probably more sensitive than grading according to K&L, however, they have their limitations in clinical and research practice. Since our intention was to develop a solid, reliable, and simply to use method to evaluate standard radiographs, KIDA was not compared to these evaluation methods but to the K&L grade, which is most commonly used to determine the severity of osteoarthritis in clinical and research settings.
A shortcoming in quantification of subchondral bone density on radiographs in general is the scattering that occurs during the radiographic imaging process (secondary radiation in the form of Compton photons). This influences the black/white intensity of the radiograph, independently of the thickness of the bone, which precludes measurement of absolute bone density values using standard radiography\textsuperscript{27}. However, to investigate the ability to detect changes in subchondral bone density using plain radiographs, the density of the bone on the radiograph was compared to the density of a step wedge reference on the same radiograph. A limitation in measurement of subchondral bone density on the standard radiographs used by KIDA was found in the fact that a maximum reliable value of bone density is reached occasionally on standard radiographs. This maximum value is calculated in the (linear) region of the values found for the step wedge. Pixels in the knee with a density value above the maximum reliable value are given the maximum value for further calculations. In these cases

Figure 4: Differences between healthy and OA knees. Mean ± SEM are depicted for healthy knees (light grey) and osteoarthritis knees (dark grey).

* Statistically significant differences in individual parameters between OA and healthy knees are depicted.
the actual bone density is underestimated and in that respect the sensitivity to detect changes in the higher bone density values decreased.

With respect to osteophyte measurement a small systematic bias was found for one observer as well as for two different observers, which makes the actual SDD larger in practice. Moreover, the value obtained is only a surrogate measure (two dimensional area) of the actual osteophyte (three dimensional) present. Also the degree of mineralization of the osteophyte will influence the measurement. However, except for one method based on microfocal radiographs described in 1991 by Buckland-Wright and colleagues\(^{28}\), KIDA is the only method that provides quantification of osteophytes as a continuous variable on standard radiographs and hence might be more sensitive to measure differences in time than the presently available grading systems. This is also indicated by the large difference in osteophyte area within one single K&L grade. Moreover, osteophyte area shows a good correlation with the K&L grade, is statistically significantly different between healthy and osteoarthritis knees, and correlates with subchondral bone density parameters (and not JSW parameters), which all together indicates that osteophyte measurement using KIDA might be of value, despite a relatively large SDD.

To our knowledge, it is still unclear whether the tibial eminences undergo changes during the development of OA and whether these changes are OA specific. KIDA gives researchers a tool to evaluate potential changes in the height of the tibial eminences in the development and progression of OA.

Sensitivity to changes has not been evaluated in the present study. Minimum joint space narrowing is identified to be \(~0.2\)mm per year, when manually measured\(^{29}\). In this respect for an individual patient follow-up, evaluation of JSW using KIDA would need at least 2 years. However, for populations this will be much less, depending on the size of the population. This corroborates that quantitative measurements of joint space narrowing have been described to be more sensitive to change than semi-quantitative measurements\(^{21}\). Thus far, there is no qualitative information on the rate of changes in subchondral bone density, osteophytes, eminence, and joint angle deviation in the process of OA. Except for lateral JSW and joint angle deviation, all parameters were statistically significantly different between healthy and OA knees. However, follow-up studies are required to see whether KIDA is indeed sensitive enough to measure changes in OA parameters in an acceptable period of time. In addition, follow-up studies are required to demonstrate whether changes in angle deviations and subluxations during the process of OA influences the reliability of KIDA, e.g. with respect to the location of the framework.

Most of the individual KIDA parameters correlated statistically significantly with the other KIDA parameters. Strikingly, within the JSW measurements, the minimum JSW correlated statistically significantly with almost all other KIDA parameters. Therefore, the minimum JSW seems to be the most sensitive JSW parameter to evaluate in osteoarthritis, as suggested before\(^{30}\) and apparently represents the process of OA in general. Additionally, a significant negative correlation was found between medial JSW and subchondral bone density at the medial side of the joint, for tibia and femur. Thus at a fixed location a lower JSW is related to a higher bone density. The presently described evaluation method provides a tool to evaluate such relations in more detail in future studies involving a larger number of OA knees with a range of disease severity.

A lot of current research in OA is focussed on biochemical markers of bone and cartilage remodelling, which are being tested to predict OA and measure disease progression\(^{31-33}\). These studies are hampered by the availability of proper imaging parameters for the ‘actual’ cartilage and bone changes. KIDA may be very helpful in this respect.

Radiography is still the golden standard for imaging of OA joints and the FDA demands radiographic changes to prove disease-modifying efficacy of treatment strategies. Therefore,
KIDA might be a worthy addition for evaluation of progression of disease in knee OA cohorts and evaluation of treatment efficacy in prospective clinical trials on knee OA.

Acknowledgments
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References


Skin and urine pentosidine weakly correlate with joint damage in a cohort of patients with early signs of osteoarthritis (CHECK).

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Abstract

**Objectives:** Age-related changes in articular cartilage are likely to play a role in the aetiology of osteoarthritis (OA). One of the major age-related changes in cartilage is the accumulation of advanced-glycation-endproducts (AGEs). Since, cartilage tissue is not readily available from patients for studying AGE levels, alternative approaches such as analysing skin and urine are needed to study the role of cartilage AGE levels in OA.

**Methods:** Paired human skin and cartilage samples were obtained *post mortem*. Paired skin and urine samples were obtained from the CHECK cohort (early OA patients). Pentosidine levels were measured by HPLC. As marker of cumulative cartilage damage X-rays of both knees and hips were scored. Urinary CTXII levels were measured, to assess current cartilage breakdown.

**Results:** Cartilage and skin pentosidine correlate well (R=0.473, p=0.05). Skin pentosidine was higher in mild (summed K&L over 4 large joints ≥4) compared to no (summed K&L≤3) radiographic OA (p=0.007). Urinary pentosidine was not different between these two groups. Skin pentosidine levels were not related to cartilage breakdown (highest versus lowest tertile of uCTXII). Urinary pentosidine, however, was higher in the highest compared to the lowest uCTXII tertile (p=0.009). Multiple regression analysis showed age to be the only predictor of the summed K&L score and age, creatinine-clearance and urinary pentosidine as predictors of uCTXII.

**Conclusion:** The higher skin and urinary pentosidine levels in those with mild compared to no radiographic joint damage and low versus high cartilage breakdown respectively suggest that AGEs may contribute to disease susceptibility and/or progression. However, relations are weak and cannot be used as surrogate markers of severity of OA.
Introduction

Osteoarthritis (OA) is the most common disease of the elderly, with a large impact on the participants quality of life\(^1\). With increasing age, the prevalence of OA increases exponentially\(^2\). In addition to chondrocyte senescence\(^3\), one of the major age-related changes in articular cartilage is the modification of proteins by non-enzymatic glycation (NEG). NEG is a common posttranslational modification of proteins caused by reducing sugars. The spontaneous condensation of reducing sugars with free amino groups in lysine or arginine residues on proteins leads to the formation of a reversible Schiff base, which is subsequently stabilized by Amadori rearrangement. Maillard reactions subsequently convert the initially formed intermediate products into advanced glycation end products (AGEs)\(^4\). As such, NEG can result in adducts to a protein, changing its properties\(^5\), but also in the formation of cross-links between proteins, changing the integrity of the cartilage matrix\(^6\). Modification by lipids is possible as well, producing advanced lipoxidation end products (ALEs)\(^7\). Moreover, NEG may change proteins of other tissues that are involved in joint function, such as bone, ligaments, and menisci\(^8\). Since these tissues, AGE accumulation is highest in cartilage due to its lowest turnover, the impact of the changes are likely most prominent in cartilage. This is because once they are formed, AGEs are only removed from the tissue when the protein involved is degraded. Articular cartilage collagen and proteoglycan proteins have an exceptionally long half-life in humans. Since the rate of AGE accumulation is largely determined by the rate of protein turnover\(^9\), this low turnover results in an abundant accumulation of AGEs in articular cartilage with increasing age\(^6,10\).

It has been reported that the accumulation of AGEs in cartilage tissue leads to inferior mechanical properties\(^6,11\) and an alteration in cartilage metabolism\(^5,12\). In vivo effects of AGEs have been identified in a canine model of experimentally induced OA by anterior cruciate ligament transection (ACLT). Animals with artificially elevated AGE levels developed more progressive OA after ACLT than did those with normal AGE levels\(^13\). Also, AGE levels in the macroscopically normal cartilage of humans with focal OA was significantly higher than in control cartilage, suggesting that elevated AGE levels predispose to OA development\(^14\).

To further understand the role of AGEs in OA, ideally cartilage biopsies would be used to assess AGE levels and relate those to OA development and/or progression in longitudinal clinical study designs. The drawback of such approach is -amongst others- the possibility of interfering with the disease process (the cartilage biopsy may induce and/or accelerate OA development). Also the effect of an ongoing disease process in which cartilage may have an increased turnover, affects the AGE levels of the cartilage biopsies\(^15\). An alternative and more feasible approach might be the use of the AGE characteristics of another, more accessible, connective tissue with relative low turnover, such as skin, as a surrogate for the AGE characteristics of cartilage. AGEs accumulation with increasing age in skin collagen is similar to that in cartilage\(^9\). Only once, the relation between skin AGEs and OA has been studied. Skin fluorescence was measured by a skin auto fluorescence reader of participants with severe OA and of a control group. No statistically significant difference was found between these two groups. The drawback of this study is that OA was in an advanced stage with presumably cartilage repair activity accompanying cartilage degeneration and the age of the participants was significant different between both groups\(^16\). Moreover, skin fluorescence is a rather rough measure for AGE accumulation.

Other surrogates for cartilage AGE levels include the analysis of AGEs in more easily accessible body fluids such as serum and urine. AGE concentrations are significantly higher in serum than in synovial fluid in participants with knee OA\(^17\). Irrespectively, previous studies have demonstrated a relation between serum AGE levels and OA. Serum pentosidine, being one of the characteristic AGE products, was significant higher in participants with OA.
Crossectional AGEs and early OA

compared to healthy controls\textsuperscript{17,18}, however no correlation with radiographic stage of the disease could be found. Baseline serum pentosidine had a significant relation to progression of OA, based on joint space narrowing\textsuperscript{18}. These results were never confirmed by other groups\textsuperscript{19}.

Urinary pentosidine, on the other hand showed different results. No significant difference in urinary pentosidine levels were found between participants with knee OA and controls\textsuperscript{20}. Urinary pentosidine levels could not predict cartilage loss (by MRI) in persons with symptomatic knee OA\textsuperscript{21}. The only relationship found with urinary pentosidine was a significant higher concentration before compared to after joint replacement surgery\textsuperscript{20}. Since urinary pentosidine levels are determined by many of the same factors as serum levels (i.e. intake, tissue degradation, and clearance), the usefulness of urinary pentosidine levels as surrogate for cartilage levels to relate to OA development might be debated.

Because of the intriguing accumulation of AGEs with increasing age and the strong relation between age and development of OA, the absence of relevant knowledge on skin AGEs as a surrogate marker for cartilage AGEs, the contradictory results for urine AGE levels when compared to serum AGE levels as marker for OA severity, and the absence of any AGE data in case of very early OA, the present study describes the presence of AGE in skin and urine in relation to OA severity, in a cohort of participants with very early signs of knee and/or hip OA (CHECK).

\textbf{Method}

\textit{Paired skin and cartilage samples}

At autopsy, paired cartilage and skin samples were obtained from 17 donors within 24 hours after death. Macroscopically normal cartilage was obtained from femoral knee condyles and humeral heads. Cartilage was cut into full thickness samples weighing 5-15 mg. In the same procedure, full thickness skin samples (10-30 mg) were taken from the incision site. All samples were stored at \(-20^\circ\text{C}\). From these paired samples pentosidine, as a marker of AGE, was measured.

\textit{Cohort description}

Part of the participants included in CHECK (the Dutch ‘cohort hip & cohort knee’) were asked to participate in the present study. From the 10 institutes that included 1002 participants, three centres (300 participants) were asked to participate in the present study. Participants were included with pain and/or stiffness in hip and/or knee, aged 45-66 years, who never or no longer than 6 month before inclusion had visited the general practitioner for these complaints. Participants with a known rheumatic condition, pain in a joint replaced by prosthesis, co-morbidity which reduces normal function, malignancy in the last 5 years, complaints started after trauma, pain in a joint that has previously been under surgery and Kellgren and Lawrence grade 4 on hip and/or knee radiograph were excluded. The researchers did not interfere with usual care with respect to advice, diagnostics nor treatment. For details see Wesseling at al.\textsuperscript{22}. The study was approved by the medical ethical committees all participating centres.

\textit{Skin samples}

All 300 participants were asked to give approval for a full-thickness punch skin biopsy (4 mm Ø). After written informed consent the biopsy was taken from the lower back under lidocain 1% and 1/100000 adrenaline local anaesthetic. Skin samples were immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\).
**Urine samples**

At inclusion and three months later a urine sample was collected and frozen at -80°C. Circadian variation of biomarker levels\(^{23}\) were not taken into account, due to logistic constraints associated with the CHECK cohort.

**AGE measurement in skin, cartilage, and urine**

Pentosidine was used as a measure of AGEs. Pentosidine in the skin and cartilage were measured as described previously\(^{10}\). Samples were reduced overnight, sequentially treated with L-Cysteine (5mM, Sigma), EDTA (50mM, Sigma), NaOH (1M, Merck) and papain (3% v/v, Sigma).

Collagen pentosidine content and amino acid composition were determined by high-performance liquid chromatography (HPLC). After hydrolysis (overnight for 20-24 hours) and subsequent drying, papain digest were dissolved in water containing the internal standards pyridoxine (10µM; Sigma) and homoarginine (2,4mM; Sigma). Samples were diluted 4 fold with 1% heptafluorobutryric acid in 10% acetonitril and analyzed by HPLC\(^{24}\). For amino acid analysis, an aliquot of the crosslink samples was diluted 200-fold with 0.1M borate buffer (pH 11.4), derivatized with 9-fluorenylmethyl chloroformate and analyzed by HPLC\(^{25}\). The pentosidine content of collagen samples is expressed as millimoles per mole collagen, assuming 300 Hyp residues per triple-helical collagen molecule\(^{25}\). A calibration curve (R\(^2\)=0.999) was used (0 to 12.5 pmol pentosidine / ml). The %CV was on average 6.8% for the various pentosidine concentrations within the calibration curve.

Urine pentosidine was measured by HPLC\(^{21}\) in diluted nonhydrolysed urine (1:1 in 0.025% H\(_2\)SO\(_4\)) following injection of 50µl on a Whatman partisik 10 SCX column (250*4.6mm). Pentosidine was eluted using an isocratic gradient of 0.025% H\(_2\)SO\(_4\) and 100 mM Al\(_2\)(SO\(_4\))\(_3\) in 0.025% H\(_2\)SO\(_4\). Column temperature was kept 60°C and eluted pentosidine detected by a Jasdo FP-1520 fluorimeter at 328nm (ex) 378nm (em). Purified pentosidine calibrated by mass spectroscopy served as a reference. Urinary creatinine was determined using the Johnson & Johnson Vitos 250 Clinical Chemistry Slides. Urinary pentosidine values were normalized by urinary creatinine to account for urinary dilution.

**Radiographic joint damage**

X-rays of both knees and hips were performed in all participants, independent of symptoms and signs. Radiographs of tibio-femoral (TF) joints were made by a weight-bearing (WB) posterior anterior (PA) view, semi-flexed (7-10°) according to Buckland-Wright\(^{26}\). For the hip, WB anterior posterior (AP) radiographs of the pelvis were made\(^{27}\). For details see Wesseling et al.\(^{22}\).

Radiographs were scored according to Kellgren & Lawrence (K&L)\(^{28}\). Total scores were calculated by taking the sum of the individual scores of the 4 joints leading to a maximum score of 16. Although a K&L grade 3 in one joint may reflect a different disease than K&L grade 1 in three joints a summed score is an accepted way to present a general burden of OA. This general burden might be related to the AGE load of a person as hypothesized. Moreover, evaluation single joints is biased by influence of explicit secondary OA in single joints, whereas the sum (or average) of the 4 larger joints means this potential variation and represents the more general OA burden.

Additionally, knee X-rays were evaluated by Knee Images Digital Analysis (KIDA)\(^{29}\) and hip X-rays by Holy (Holy’s software-β19/20™)\(^{30}\). These are computer assisted programs that semi-automatically calculates, mean and minimum joint space width (JSW). The sum of the minimum, mean (lateral and medial compartment), and smallest mean (lateral or medial compartment) JSW of 4 joints was used for further analysis. In total 22 X-rays were excluded due to technical problems (inferior X-ray quality did not allow KIDA analysis). MTP alignment was used for all knee X-rays.

**Cartilage breakdown; uCTXII**
CTXII as a biomarker specific for degradation / turnover of hyaline cartilage type II collagen was measured by ELISA (CartiLaps, Nordic Bioscience, Herlev, Denmark). Intra-assay and inter-assay variation of the same sample was less than 9% and 11%, respectively.

Kidney function
As a potential confounder, kidney function was determined. Creatinine in peripheral blood was measured by Unicel DxC 800, Beckman Coulter (Fullerton, Ca, USA). Estimated glomerular filtration rate was calculated by the Cockroft-Gault formula (creatinine clearance=[(140-age)*weight/](0.84*serum creatinine)*0.85 if subject is female). Creatinine clearance is expressed in millimetres per minute per 1.73m$^2$.

Statistical analysis
Since the measurements of pentosidine were not normally distributed non parametric statistical test were used. Differences between groups of summed K&L (i.e. sum of K&L grade of the two knees and hips) were first described and tested by a non-parametric Mann Whitney U test. P-values <0.05 were considered statistically significant.

Subsequently, non-parametric Spearman correlations were calculated for the association between measures of pentosidine and total joint damage (measured as the sum of K&L scores in all 4 joints). Multiple regression analysis was used to describe the association between the measures of pentosidine and joint damage and to evaluate the influence of multiple participant characteristics (confounders) in addition to pentosidine levels on total joint damage. Subgroup analyses for the hips and the knees were performed as well. SPSS 15.0 software was used.

Figure 1: The correlation between cartilage and skin pentosidine. Paired cartilage and skin samples were obtained post-mortem of 17 donors. Cartilage was obtained from femoral knee condyles and humeral heads, skin samples were taken from the incision site. Regression coefficient and p value have been provided.
Results

Skin versus cartilage pentosidine

Pentosidine as a marker for AGE levels was measured in post mortem cartilage and skin samples of the same individuals. A statistically significant correlation was found between these 17 paired cartilage and skin pentosidine levels (R=0.473, p=0.05; figure 1). Pentosidine levels were normalised to collagen amount and a regression coefficient of 0.19 for the association between skin and cartilage pentosidine was found. This can be interpreted as an increase in skin pentosidine per unit increase of cartilage pentosidine of 0.19 points (i.e. as such might be concluded that the accumulation of pentosidine is ~5 times higher for cartilage than for skin), consistent with previously unpaired data\textsuperscript{10}.

Table 1: characteristics of the 205 participants included in the cohort. Variables are displayed in mean ± SD (minimum-maximum) if applicable.

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<tr>
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<tr>
<td>skin pentosidine/collagen (mmol/mol)</td>
<td>2.42 ± 0.79, (0.948-5.685)</td>
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<tr>
<td>urine pentosidine/creatinine (µmol/mol)</td>
<td>0.57 ± 0.18 (0.26-1.58)</td>
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<td>CTXII (µg/mmol)</td>
<td>0.32 ± 0.18 (0.03-0.99)</td>
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<tr>
<td>creatinine clearance (cockroft gault; ml/min)</td>
<td>93.4 ± 21.5 (52.0-170)</td>
</tr>
<tr>
<td>BMI</td>
<td>26.8 ± 3.8 (15.4-40.4)</td>
</tr>
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</table>

| summed mean JSW of both knees (mm) | 10.13 ± 2.05 (4.51-16.26) |
| summed minimum JSW of both knees (mm) | 6.36 ± 1.89 (0.03-10.26) |
| summed mean JSW of both hips (mm)  | 8.16 ± 1.40 (2.97-12.27) |
| summed minimum JSW of both hips (mm) | 7.13 ± 1.41 (1.21-11.53) |
| summed mean JSW of all 4 joints (mm) | 18.43 ± 2.82 (12.09-26.29) |
| summed min JSW of all 4 joints (mm)  | 13.58 ± 2.36 (6.96-18.80) |

<table>
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<th>3</th>
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<td>6</td>
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<th>Summed K&amp;L grade of 4 joints</th>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
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<tr>
<td>Number of patients</td>
<td>67</td>
<td>57</td>
<td>34</td>
<td>15</td>
<td>21</td>
<td>3</td>
<td>4</td>
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<td>2</td>
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</tbody>
</table>

| Dichotomous numbers | 173 | 32 |
Characteristics of the study population.
Three hundred participants from the 3 different medical centres were asked to participate in this study, 244 signed informed consent. From these 244 participants complete data sets (skin and urine pentosidine levels, age, BMI, creatinine clearance, and X-ray data of the 4 joints) were available of 205 participants. The major loss of participants was due to missing urine pentosidine data on both time points (49). In 19 cases the only available single sample was used. In table 1 the characteristics of these 205 participants are given. Note that although most participants fulfilled the ACR criteria for clinical OA for at least one joint (65%) this is a cohort with no to mild radiographic joint damage (68% and 26% K&L grade 0 and 1, respectively of the 820 joint radiographs). Mean JSW varied between 1.61 and 8.43 mm for knee and 1.21 and 6.66 for hip.

Skin pentosidine related to radiographic joint damage
Participants were divided in two groups based on radiographic joint damage, participants with a summed K&L score of the 4 joints ≤3 and ≥4, further indicates as ‘no’ and ‘mild’ radiographic joint damage, respectively. In the group with mild radiographic joint damage slightly (but statistically significant) higher skin pentosidine levels were found (figure 2A). Because cartilage volume of the knee is larger than of the hip, separate evaluations for knee and hip were made. In these cases the cut-off for no and mild radiographic damage was set on ≤1 and ≥2, respectively. For knee OA a higher skin pentosidine level in the group with mild radiographic joint damage was observed compared to the subjects without OA. This difference was not found when participants were grouped based on severity of hip OA (figure 2B and 2C, respectively).

Figure 2: Correlation of skin pentosidine and the summed K&L score of the 4 joints. The summed K&L score was divided into 2 groups, no (summed K&L score 0-3; n=173) and mild (summed K&L score 4-12; n=32) radiological OA. Joint with K&L score 4 were not included. Participants with low radiological OA had a lower skin pentosidine compared to the participants with mild radiological OA (A). Separating the knees (B; summed K&L score 0-1 or 3-6; n=178 and 27, respectively) and hips (C; summed K&L score 0-1 or 3-6; n=138 and 67, respectively) shows that only for the knees this difference is maintained. Median (dash), with the interquartile range (box; 50%) with the 5th and 95th percentiles (whiskers) are presented. (D) The correlation between skin pentosidine and summed K&L score of the 2 knees (n=205). Spearman correlation coefficient with p value has been provided.
Although in dichotomous categories there was a difference in skin pentosidine for those participants with the summed K&L score ≥4 versus the group with a score of ≤3, using Spearman rank correlation no correlations between skin pentosidine and summed K&L scores were found. Only in case of the knee a tendency toward a significant correlation was observed (R=0.122, p=0.082) (figure 2D).

When joint space widths (minimal, mean and smallest mean) were measured as continuous variable by use of digital analyses of the radiographs (KIDA and Holy) no correlations were found between skin pentosidine levels and the sum of the mean and minimum JSW of the 4 joints, nor when knees and hips were taken separately (data not shown).

**Skin pentosidine related to cartilage breakdown**

In case levels of CTXII (a biochemical marker of cartilage breakdown) were used as an outcome parameter for cartilage breakdown, no difference were found for skin pentosidine between patients with different levels of uCTXII, analysed as continues as well as ordinal variable (data not shown).

**Skin versus urine pentosidine**

Because of diet-induced variation in urine NEG and with that pentosidine levels, pentosidine in urine was measured twice, at inclusion and after 3 months (mean difference of these 2 measurements 17 nmol/mol; 95% confidence interval -5.5-39.6 nmol/mol). The mean of these two AGE levels was used for further calculations. There was a small but statistically significant correlation between skin and urinary pentosidine (R=0.285, p=0.001; figure 3).

![Figure 3: The correlation between mean urine pentosidine and skin pentosidine of 205 participants. There is a statistically significant correlation between both parameters, with a low correlation coefficient.](image)

**Urine pentosidine related to radiographic joint damage**

No difference could be found for urine pentosidine between the participants with no versus mild radiographic joint damage (data not shown). Separate evaluations for knee and hip radiographic damage did not provide a difference in urine pentosidine levels between the groups (data not shown). No correlations between urine pentosidine and summed K&L scores
were found. When joint space widths (minimal, mean, and smallest mean) were measured digitally as continuous variables, no correlations were found with urine pentosidine levels either (data not shown).

**Urine pentosidine related to cartilage breakdown**

Urine pentosidine levels on the other hand differ between patients within the lowest tertile of uCTXII levels from those within the middle and highest tertile CTXII levels (p=0.033 and 0.009 respectively; figure 4A). Also a correlation was found between urine pentosidine and CTXII a measure of cartilage breakdown (R=0.235 p=0.001; figure 4B).

![Figure 4](image_url)

**Figure 4:** Correlation of mean urine pentosidine (A) and urine CTXII levels divided into 3 groups, low (≤ 0.22 µg/mmol; n=65), middle (0.22-0.37 µg/mmol; n=66) and high (≥0.37 µg/mmol; n=66) CTXII levels). For urine pentosidine all groups were different except the middle and high CTXII group. Median (dash), with he interquartile range (box; 50%) with the 5th and 95th percentiles (whiskers) are presented. Figure B shows the correlation between urine pentosidine and CTXII levels. A low correlation between these parameters was found (R=0.235, p=0.001; n=200).

**Influence of other factors related to K&L**

In addition to skin and urine pentosidine levels several participant/patient characteristics are known to influence K&L score or uCTXII levels, including factors such as age, BMI and, gender. For serum pentosidine it is known that age and creatinine clearance are major influencing factors. Table 2 shows the results of the univariate linear regression analyses with total joint damage as outcome variable. A statistically significant relation between creatinine clearance, age, and gender and total joint damage was found, for all other parameters no relation was found. In multivariable regression analysis (table 2) with skin pentosidine, mean
urine pentosidine, creatinine clearance, age, BMI, and gender as independent variables and summed K&L as dependent variable the explained variance was low ($R^2=0.068$) indicating that all parameters explain 6.8% of the summed K&L score. The partial regression coefficient for creatinine clearance and age showed a tendency to statistically significant (p=0.099 and 0.067 respectively).

In case of uCTXII as outcome parameter of cartilage breakdown (table 2) with skin pentosidene, mean urine pentosidine, creatinine clearance, age, BMI, and gender as independent variables the explained variance was intermediate ($R^2=0.396$) indicating that all parameters explain ~40% of the CTXII level. The partial regression coefficient for urine pentosidine level, age, and creatinine clearance was statistically significant. Multivariate analysis for skin and urine separately with summed K&L score and uCTXII respectively show comparable results.
Table 2: The regression of different parameters with summed K&L score in univariate regression analysis. Age, creatinine clearance, and gender show a statistically significant regression with summed K&L score. The β coefficients and p values for multivariate regression analysis with skin pentosidine, mean urine pentosidine, BMI, age, creatinine clearance, and gender as independent variables and summed K&L score and CTXII as outcome measurement are indicated. Multivariate regression with only skin or urine pentosidine is included as well.

<table>
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<tr>
<th></th>
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<th>sum K&amp;L</th>
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<tr>
<td></td>
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<td>β</td>
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Crossectional AGEs and early OA
Discussion
This is the first study describing the relationship between skin and urine pentosidine with severity of joint damage in early OA, using radiographic OA and uCTXII as markers of cumulative cartilage damage and current breakdown, respectively. In this cross-sectional study a small, but statistically significant, increase in skin pentosidine levels was observed in participants with mild versus no radiographic joint damage. Urinary pentosidine was not correlated with radiographic OA. For urine pentosidine levels a weak correlation was found with cartilage breakdown as measured by uCTXII, but not for skin pentosidine.

Skin and cartilage share several characteristic influencing AGE formation. Tissue glucose concentration and oxidative stress are relatively similar for cartilage and skin collagen. Both cartilage and skin collagen AGE formation are presumably dependent on glucose levels and not on other sugars. However, cartilage and skin collagen have a different protein turnover rate. Cartilage collagen does hardly renew during life, whereas skin collagen has a turnover faster than cartilage. This was also clear from our study; cartilage pentosidine is ~5 times higher compared to skin pentosidine levels in paired samples. This fits well with the slow turnover of collagen in cartilage compared to skin and consequently a higher rate of AGE accumulation in cartilage compared to skin. Using skin pentosidine levels as a surrogate of cartilage pentosidine a weak but statistically significant relation with severity of radiographic OA was found. Assuming cartilage AGE levels to be directly related to OA a stronger relation was expected. However, the observed variance between cartilage and skin pentosidine levels limits the usefulness of skin AGE levels as a representative of cartilage AGE levels and as such hampers studies on the causality of the relationship. Furthermore, in case of the dichotomous division into ‘no’ versus ‘mild’ radiographic damage, age was different between both groups for the total as well as for the knee separately (p=0.05 and 0.02 respectively). As such the difference in skin pentosidine levels can also be explained by the difference in age between both groups. This dominance of age in pentosidine levels was also found in the multiple regression analyses (table 2).

It should be noted that the use of X-rays in general and most specifically in the present cohort with very early OA has its limitations. The K&L score is a poor indicator of articular cartilage damage, in the early phases of the disease. Also joint space narrowing (JSN) in the knee may be influenced by many factors other than cartilage damage including meniscus extrusion, subluxation, poor alignment of the medial tibial plateau (resulting in suboptimal measurements), hypertrophic cartilage repair (increasing the JSW), etc. These confounders may obscure the detection of a potential relationship between cartilage composition (i.e. AGE level) and OA.

In the present study, no differences in urinary pentosidine between no and mild OA based on radiographic OA was found, an outcome that corroborates other studies. On the other hand, urine pentosidine correlated with uCTXII as a marker of cartilage breakdown. In multivariable regression analyses this was still evident in addition to age and creatinine clearance, suggesting that urinary pentosidine may more reflect current cartilage breakdown than overall AGE level. Skin pentosidine did not correlate with uCTXII. Since both the urinary pentosidine and the uCTXII are excreted via the kidney, kidney function may limit variations due to individual differences in processing and by that can explain that this correlation was found for urinary pentosidine and not for skin pentosidine.

The fact that skin pentosidine correlates with radiographic OA and urine pentosidine does not might be explained by the fact that skin pentosidine is a marker of the lifelong accumulation of AGEs in tissue, similar to the accumulation of AGEs in cartilage tissue. Urinary pentosidine is measured in a momentum (as is uCTXII) dependent on various factors including peripheral turnover. In general it should be noted that pentosidine is used as a biomarker for the whole class of AGEs. Other AGEs such as carboxymethyllysine could be
simply used for this purpose. It cannot be excluded that other relations would have been found when other markers would have been analyzed.

In general there are numerous confounding factors in finding a relation between surrogate markers of cartilage AGE levels (skin and urine pentosidine) and cartilage damage (JSW on X-ray and urine CTXII). The complexity between cartilage, bone, synovial tissue, ligaments and muscle strength all determine cartilage damage and are not directly related to cartilage AGE levels. Moreover, AGEs by themselves do not cause OA, rather they predispose to the development of the disease. A trigger, most likely mechanical of nature, is needed to induce the disease process. Accumulation of AGEs increase the susceptibility of articular cartilage to damage due to micro-traumata over a lifetime. In other words, AGE accumulation makes the joint more prone to develop diseases.

It has been demonstrated that nearly one-third of human knees from donors in their 7th - 9th decade who had no history of arthritis, showed no evidence of OA. According to this hypothesis, elderly without OA either have low AGE levels, or have high AGE levels, but did never encounter a sufficiently strong mechanical trigger that induced the disease. The latter however is difficult to verify.

In the present study we specifically choose for very early joint degeneration. In more advanced stages breakdown of cartilage might be accompanied by repair activity and breakdown of repaired tissue, the latter with relative low levels of accumulated AGEs. In these more advanced stages the complex turnover rate of cartilage matrix might disturb a potentially clear correlation. This is supported by the fact that Matsumoto and co-workers did not find enhanced skin AGE levels in advanced stage OA, whereas the present study demonstrated this, although rather weak.

In conclusion, the current data support earlier observations that urinary pentosidine levels are not of use as surrogate markers of severity of cartilage breakdown in clinical studies, despite its correlation with another marker of cartilage breakdown, CTXII. However, our data show that skin pentosidine (which is unlikely to be affected by tissue remodelling due to the OA disease process) is statistically significantly increased in participants with mild OA compared to those without radiographic OA. As such these observations still support that AGE accumulation may be one of the molecular mechanisms by which aging leads to OA. However, relations are weak and age is still a confounder.

Conflict of interest
The authors report no conflict of interest.

Author contribution
All authors have made substantial contribution to the study design or acquisition of data, drafting of the article and final approval of the version submitted.

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References


Skin pentosidine in very early hip/knee osteoarthritis (CHECK) is not a strong independent predictor of radiographic progression over 5 years follow-up.

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Welsing PMJ\textsuperscript{1,8}
deGroot J\textsuperscript{2}
Huisman AM\textsuperscript{3}
Oostveen JCM\textsuperscript{4}
Reijman M\textsuperscript{5}
Bierma-Zeinstra SM\textsuperscript{6}
Mastbergen SC\textsuperscript{1}
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Abstract

Objectives: Age-related changes in articular cartilage are likely to play a role in the aetiology of osteoarthritis (OA). One of the major age-related changes in cartilage is the accumulation of advanced-glycation-endproducts (AGEs). Cross-sectionally we showed that skin pentosidine, as a marker of cartilage AGE, is higher in participants with mild compared to no OA. The present study evaluates whether skin pentosidine can predict radiographic OA progression and/or radiographic OA burden over 5 years follow-up.

Methods. The 5 years follow-up data of 300 patients from the very early OA cohort CHECK were used. Radiographic progression and burden were assessed by X-rays of both knees and hips scored according to K&L and Altman atlas (joint space narrowing (JSN) and osteophytes). Burden and progression scores were defined using the Area Under the Curve (AUC), change between T0 and T5 (progression) and status at T5 (burden). Baseline skin pentosidine levels were measured by HPLC. Levels of uCTXII as a comparator, were measured to assess baseline joint tissue breakdown. Univariate associations of skin pentosidine and uCTXII with radiographic burden/progression were studied and also multivariate associations also including baseline damage, age, gender, BMI and kidney function as predictors.

Results: Both skin pentosidine and uCTXII correlated with radiographic progression and burden. In general skin pentosidine did not have an added predictive value to uCTXII for progression or burden of the disease. The best prediction was obtained for burden of radiographic damage (R²=0.60-0.88), bus this was predominantly determined by baseline radiographic damage. Without this parameter predictive ability of these models decreased significantly (R²=0.07-0.17), to a level seen for radiographic progression (R²=0.03-0.23). Interestingly, skin pentosidine significantly added to prediction of osteophyte formation, whereas uCTXII significantly added to prediction of JSN.

Conclusion: Skin pentosidine adds to prediction of radiographic progression and burden of osteophyte formation and uCTXII to radiographic progression and burden of JSN, but overall skin pentosidine did not add to uCTX in predicting radiographic progression or burden. Burden of damage over 5 year is mainly determined by damage at baseline.
Introduction
The pathogenesis of osteoarthritis (OA) is unknown. Several risk factors are described, age being clearly one of the main factors. A major age-related change in articular cartilage is the modification of proteins by non-enzymatic-glycation (NEG). NEG is a common posttranslational modification of proteins caused by reducing sugars. The spontaneous condensation of reducing sugars with free amino groups in lysine or arginine residues on proteins leads to the formation of a reversible Schiff base, which is subsequently stabilized by Amadori rearrangement. Maillard reactions subsequently convert the initially formed intermediate products into advanced glycation end products (AGEs)\(^1\). As such, NEG can result in adducts to a protein, changing its properties\(^2\), but also in the formation of cross-links between proteins, changing the integrity of the cartilage matrix\(^3\). Modification by lipids is possible as well, producing advanced lipoxidation end products (ALEs)\(^4\).

AGEs are only removed from tissues when the proteins involved are degraded and removed. Cartilage tissue has a low turnover of its proteins resulting in abundant AGE accumulation\(^3,5\). *In vitro* investigations show that AGEs have a negative influence on mechanical\(^3,6\) and biochemical\(^2,7\) properties of the cartilage tissue making it more prone to mechanical damage. *In vivo* effect of AGE have been investigated in several models. In the anterior cruciate ligament transaction (ACLT) model artificial elevated AGE levels of the joint cartilage leads to an increase in progression of OA compared to joints without elevated AGE levels\(^8\). Upon minimal applied surgical damage of the cartilage according to the canine Groove model\(^9\) in combination with restricted joint loading, artificial increase of AGE levels shows a tendency to more severe OA progression\(^10\).

To further understand the role of AGEs in specifically human OA, ideally cartilage biopsies would be used to assess AGE levels and relate those to OA development and/or progression in longitudinal study designs. The drawback of such an approach is -amongst others- the possibility of interfering with the disease process (the cartilage biopsy may induce and/or accelerate OA development). An alternative and more feasible approach might be the use of the AGE characteristics of another, more accessible, connective tissue with relative low turnover, such as skin, as a surrogate for the AGE characteristics of cartilage. AGE accumulation with increasing age in skin collagen is related to accumulation in cartilage\(^5,11\). Cartilage pentosidine is about 5 times higher compared to skin pentosidine\(^11\). When using skin pentosidine as a surrogate marker for cartilage pentosidine in a cross sectional study on early radiographic OA, skin pentosidine is increased in patients with mild OA compared to those without OA\(^11\). As such skin AGE levels might be considered a biochemical marker for radiographic OA, although not of use at an individual patients’ level.

Urine CTXII is from all biomarkers reported on thus far, the one that best relates to development and progression of osteoarthritis\(^12,13\). Moreover, this biomarker has already been proven applicable in clinical trials to monitor disease activity and to decide on treatment of rheumatoid arthritis\(^14\). As such this biochemical marker is a relevant comparator in case of studies on biochemical markers of joint damage.

Up till now plain radiographs are the gold standard for imaging hip and knee joint damage. These radiographs can be scored in different ways. The Kellgren and Lawrence classification\(^15\) is probably the most used one. This method provides an integrated score using joint space narrowing (JSN), osteophyte formation, and subchondral sclerosis in a combined score. Additional scores have been developed to provide a subcategorising of these individual radiographic features, i.e. the Altman atlas score\(^16\), that scores JSN and osteophytes independently. Both these methods use a stepwise scoring (ordinal variable) (e.g 0-4 for K&L and max 0-3 for JSN for Altman).

As mentioned above, in a cross sectional study we showed in a cohort of patients with very early signs of knee and/or hip OA (CHECK) that skin pentosidine was higher in participants
with more severe radiographic joint damage as calculated by the sum of the K&L score of both hips and knees\textsuperscript{11}. The present study describes whether these skin pentosidine levels are useful to predict progression of OA over 5 years follow-up, compared to / or in addition to uCTXII, also taking demographic and radiographic baseline characteristics into account.

**Method**

*Cohort description*

From the 1002 participants included in the Dutch CHECK cohort (‘cohort hip & cohort knee’) 300 participants (3 of the 10 participating institutes) were asked to participate in the present study. Participants were included with pain and/or stiffness in hip and/or knee, aged 45-66 years, who never or no longer than 6 month before inclusion had visited the general practitioner for these complaints. Participants with a known rheumatic condition, pain in a joint replaced by prosthesis, co-morbidity which reduces normal function, malignancy in the last 5 years, complaints started after trauma, pain in a joint that has previously been under surgery, and Kellgren and Lawrence grade IV on hip and/or knee radiograph were excluded. The researchers did not interfere with usual care with respect to advice, diagnostics nor treatment. For details see Wesseling at al.\textsuperscript{17}.

*Skin pentosidine as surrogate markers of cartilage AGE*

At baseline a full-thickness punch skin biopsy (4 mm Ø) was taken of all those participants that gave additional informed consent\textsuperscript{11}. The CHECK study and this additional study were approved by medical ethical committee of the UMC Utrecht and according to the declaration of Helsinki. All participants gave written informed consent. Pentosidine was used as a measure of the various AGEs formed. It is reported that pentosidine represents a number of AGE markers such as N\textsuperscript{ε}-(carboxymethyl)lysine and N\textsuperscript{ε}-(carboxyethyl)lysine\textsuperscript{18}. Pentosidine in the skin was measured by HPLC as described previously\textsuperscript{11}. In short: Samples were reduced overnight, sequentially treated with L-Cysteine (5mM, Sigma), EDTA (50mM, Sigma), NaOH (1M, Merck) and papain (3% v/v. Sigma). Collagen pentosidine content and amino acid composition were determined by high-performance liquid chromatography (HPLC). After hydrolysis (overnight for 20-24 hours) and subsequent drying, papain digest were dissolved in water containing the internal standards pyridoxine (10μM; Sigma) and homoarginine (2,4mM; Sigma). Samples were diluted 4 fold with 1% heptafluorobutyric acid in 10% acetonitril and analyzed by HPLC\textsuperscript{19}. For amino acid analysis, an aliquot of the crosslink samples was diluted 200-fold with 0.1M borate buffer (pH 11.4), derivatized with 9-fluorenlymethyl chloroformate and analyzed by HPLC\textsuperscript{20}. The pentosidine content of collagen samples is expressed as millimoles per mole collagen, assuming 300 Hyp residues per triple-helical collagen molecule\textsuperscript{20}.

*Cartilage breakdown marker; uCTXII*

uCTXII as a biomarker supposed rather specific for degradation of hyaline cartilage type II collagen was measured by ELISA (CartiLaps, Nordic Bioscience, Herlev, Denmark) fully according to manufacturer’s guidelines. Intra-assay and inter-assay variation of the same sample were both <= 10%. Urinary creatinine was determined using the Johnson & Johnson Vitos 250 Clinical Chemistry Slides. Urinary pentosidine values were normalized by urinary creatinine to account for urinary dilution. uCTXII is expressed as nano gram per mmol creatinine.
Kidney function
As a potential confounder, kidney function was determined since it might be related to skin pentosidine level and uCTXII due to the fact that less excretion of plasma pentosidine and CTXII in kidney dysfunction is seen\textsuperscript{21} and might also be related to more severe disease. Creatinine in peripheral blood was measured by Unicel DxC 800, Beckman Coulter (Fullerton, Ca, USA). Estimated glomerular filtration rate was calculated by the Cockroft-Gault formula (creatinine clearance=[(140-age)*weight]/[0.84*serum creatinine]*0.85(if subject is female). Creatinine clearance is expressed in millimetres per minute per 1.73m\textsuperscript{2}\textsuperscript{22}.

Radiographic joint damage
Radiographs of both knees and hips were taken from all participants, independent of symptoms and signs. Radiographs of tibio-femoral (TF) joints were made by a weight-bearing posterior anterior view, semi-flexed (7-10\degree) according to Buckland-Wright\textsuperscript{23}. For the hip, weight-bearing anterior posterior radiographs of the pelvis were made\textsuperscript{24}. For details see Wesseling et al.\textsuperscript{17}.
Radiographs of both knees and hips were obtained at baseline (T0) and after 2 and 5 years (T2 and T5). All radiographs were scored according to Kellgren & Lawrence\textsuperscript{15} in a paired fashion (all three time points in view at the same time).
Radiographic OA burden was expressed as the summed K&L grade of both hips and knees. When a prosthesis was implanted (in 12 cases) the score+1 (unless already maximum) of the latest time point was used. Total scores were calculated by taking the sum of the individual scores of the 4 joints leading to a maximum score of 16. Also the different radiographic features were scored by using the Altman atlas\textsuperscript{16}. The Altman score provides scores of the joint space narrowing (JSN; 0-3) at 2 places for the knees and 2 for the hips. The sum of these scores was used to measure the overall JSN of 4 joints (max. 24). The osteophyte formation is scored on 4 different places in the knee as well as the hip (0-3 except for acetabulum inferior 0-1). The sum of these scores was calculated for a total score of the osteophyte formation of a patient (max 44).
Radiographic OA progression was expressed as the absolute progression of the summed K&L grade between baseline and 5 years (i.e. summed K&L grade at 5 years minus summed K&L grade at baseline; T5-T0) and as the area under the curve (AUC) of the summed K&L grade between baseline and 5 years (i.e. according to the formula (summed K&L grade at 2 years minus the one at baseline) *2 (years) *0.5 (slope) + (summed K&L grade at 5 years minus the one at 2 years) *3 (years) *0.5 (slope).
The burden of radiographic joint damage was expressed as the summed K&L grade at 5 years (T5), and as the AUC as described above plus the total score at baseline * 5 (years). For the summed Altman scores for JSN and osteophyte formation similar formulas were used to determine progression and burden scores per patient.

Statistical analysis
Continuous variables were described using means with standard deviation or medians with 25\textsuperscript{th} and 75\textsuperscript{th} percentiles where appropriate, categorical data were described using frequencies and percentages.
Wilcoxon rank tests for related samples were used to test differences between T0 and T5 regarding the different radiographic scores (K&L and Altman JSN and osteophytes).
Skin pentosidine and uCTXII were divided in three equal groups (tertiles) and the radiographic progression as well as burden scores were compared between the group with the highest and the lowest biomarker level and tested using Mann Whitney U tests.
Correlations of skin pentosidine levels as well as uCTXII levels with radiographic progression and burden scores were investigated by Pearson correlation.
Multivariate linear regression analyses were used to correct for possible confounders and investigate the additional predictive value of skin pentosidine/uCTXII above other predictors. In these analyses age, gender, creatinine clearance, and BMI as well as baseline radiographic scores were evaluated as covariates. Final models were defined using a backward selection strategy starting with all variables in the model and removing variables that were not statistically significantly related to the outcome or confounders for the relation of skin pentosidine/uCTXII with outcome (based on a change in the regression coefficient of >10%) one by one.

To facilitate comparison between regression coefficients within and between models, regression coefficients were expressed as standardized betas. Standardized betas represent the number of standard deviations that the outcome will change as a result of one standard deviation change in the predictor and are therefore independent of the units of measurement of the variables and can vary between -1 and +1.

For use in the Pearson correlation and regression analyses, progression and burden scores for JSN and osteophytes were transformed by taking the square root, to achieve a normal distribution. The assumptions for the regression analysis were checked graphically using a histogram and normal probability plot of the residuals. The observed and predicted outcomes were plotted against each other to check for heteroscedasticity. A $p$ value $\leq 0.05$ was taken as statistically significant and all statistical analyses were performed using SPSS version 15.0.

Results

Characteristics of study population

The characteristics of the study population and of the radiographic progression are shown in table 1A and B, respectively. Of the 300 patients asked for informed consent, 56 participants did not participate in this study. In 61 cases radiographic data were not complete for all three time points and/or biochemical data were missing, so for 183 participants all data were available and these were used in the current analyses. The baseline demographics (age, gender, BMI, and kidney function) and baseline radiographic characteristics did not differ statistically from the original 244 cohort patients. For all radiographic parameters statistically significant progression of OA was seen (table 1B).

Table 1A: The baseline characteristics of the study population.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean values ±SD with min and max values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.5 ± 5.4</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26.37</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>30/153</td>
</tr>
<tr>
<td>Skin pentosidine/collagen (mmol/mol)</td>
<td>2.34</td>
</tr>
<tr>
<td>uCTXII (ngr/mmol)</td>
<td>0.29</td>
</tr>
<tr>
<td>Creatinine clearance (ml/ min/1.73m$^2$)</td>
<td>93.8 ± 22.1</td>
</tr>
</tbody>
</table>

$^1$Mean values +SD with min and max values; $^2$median values with 25-75 percentiles; $^3$actual numbers of patients. BMI= body mass index
AGEs and radiographic OA progression and burden

Table 1B: The radiographic characteristics at baseline and 5 years follow-up. Sum K&L score calculated by taking the sum of the individual scores of the 4 joints leading to a maximum score of 16. Sum Altman JSN and osteophyte score is the sum of the scores of the different locations (2 and 4, respectively) in all 4 individual joints (max. 24 and 44, respectively).

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th></th>
<th>T5</th>
<th></th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>min-max</td>
<td>mean ± SD</td>
<td>min-max</td>
<td>T0 – T5</td>
</tr>
<tr>
<td>sum K&amp;L score</td>
<td>1.69 ± 1.13</td>
<td>0 – 4</td>
<td>3.30 ± 1.81</td>
<td>0 – 7</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>sum Altman JSN</td>
<td>3.40 ± 2.58</td>
<td>0 – 11</td>
<td>4.97 ± 3.02</td>
<td>0 – 14</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>sum Altman osteophyte</td>
<td>3.34 ± 3.01</td>
<td>0 – 15</td>
<td>6.47 ± 4.52</td>
<td>0 – 22</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Differences in radiographic progression and burden between low and high skin pentosidine as well as low and high uCTXII

To test whether patients with high versus low skin pentosidine/uCTXII levels at baseline had a different progression and/or total burden of radiographic OA over 5 years, the lowest and highest tertiles of skin pentosidine/uCTXII were compared for radiographic progression and burden scores (figure 1).

Figure 1: Difference between progression and burden of radiographic OA for participants with low and high skin pentosidine, uCTXII or the combination.

Participants with low (white) and high (dashed) tertiles skin pentosidine (A-D; n=66 in the low and high group), urine CTXII (E-H (n= 68 and 67 in the low and high group resp), and both (I-L; n=21 and 17 in the low and high group, respectively) at baseline. Differences in sum K&L (A, D, G), sum Altman JSN (B, E, F), and sum Altman osteophytes (C, F, G) scores are given. Median (dash), with the interquartile range (box; 50%) with the fifth and ninety-fifth percentiles (whiskers) are presented. P values are indicated.
For skin pentosidine the AUC progression and burden sum K&L score showed no difference between patients with the highest and lowest tertile (figure 1A). The same was found for AUC progression and burden Altman JSN (figure 1B). For Altman osteophytes the AUC progression and burden were significantly higher in the group with high skin pentosidine (figure 1C; for progression and burden, \( p=0.001 \) and 0.024, respectively).

For the highest and lowest uCTXII tertiles, all AUC progression and burden scores (figure 1D, figure 1E and F) were statistically significant different, with the most severe damage scores for the highest uCTXII level (except for progression of JSN AUC, \( p=0.126 \)). When the participants with low skin pentosidine and low uCTXII were compared with those for both parameters in the highest tertile (\( N=21 \) and 17 for both lowest and both highest combined tertiles, respectively) all radiographic characteristics were more severe in the group with high compared to low combined pentosidine and uCTXII values (figure 1 G-I) and differences became clearly more outspoken, suggesting additive predictive ability.

In all cases when progression and burden of radiographic damage over 5 year time were expressed as T5-T0 and T5, respectively, instead of AUC progression and burden, data were very similar and statistical significance were the same (data not shown).

### Table 2: Correlations between skin pentosidine levels and uCTXII levels with radiographic progression of OA and burden of OA.

<table>
<thead>
<tr>
<th>Progression or Burden</th>
<th>Skin pentosidine AUC</th>
<th>Skin pentosidine</th>
<th>uCTXII</th>
<th>uCTXII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum K&amp;L</td>
<td>0.167</td>
<td>0.024</td>
<td>0.323</td>
<td>0.000</td>
</tr>
<tr>
<td>Sum Altman JSN</td>
<td>0.123</td>
<td>0.095</td>
<td>0.190</td>
<td>0.016</td>
</tr>
<tr>
<td>Sum Altman osteophytes</td>
<td>0.301</td>
<td>0.000</td>
<td>0.194</td>
<td>0.014</td>
</tr>
<tr>
<td>Burden (total) AUC</td>
<td>0.056</td>
<td>0.454</td>
<td>0.360</td>
<td>0.000</td>
</tr>
<tr>
<td>Sum K&amp;L</td>
<td>0.113</td>
<td>0.127</td>
<td>0.332</td>
<td>0.000</td>
</tr>
<tr>
<td>Sum Altman osteophytes</td>
<td>0.180</td>
<td>0.015</td>
<td>0.290</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Correlation of skin pentosidine and uCTXII with radiographic damage**

In table 2 correlations of skin pentosidine as well as uCTXII with radiographic progression and burden for the overall (K&L) and separate characteristics (JSN and osteophytes) calculated as AUC are given. In general, correlations with radiographic progression and burden were stronger for uCTXII than for skin pentosidine. For uCTXII relations with radiographic burden were in general stronger than for radiographic progression. For skin pentosidine this was the other way around.

The results of the analyses based on AUC progression and burden scores were supported by the other results of the analyses based on progression and burden scores based on T5-T0 and T5 data, not shown.

Figure 2 provides representative figures for skin pentosidine against sum K&L grade for progression and burden (A and B, respectively) and for uCTXII for sum Altman JSN for progression and burden (C and D, respectively).

**Multiple regression analysis**

In table 3 different final regression models based on the multivariate linear regression analysis are shown. \( R^2 \) for the models are given as well as standardised betas and \( p \) values for the different independent variables.

For the progression scores (calculated by AUC) for all 3 radiographic outcome parameters (sum K&L, Alman JSN, and Alman osteophytes) the explained variances (\( R^2 \)) were low, between 0.03-0.23. For the radiographic burden scores the \( R^2 \) are higher, between 0.69-0.88.
Correlations skin pentosidine and uCTXII with parameters of radiographic progression and burden.

For skin pentosidine the sum K&L scores for progression and burden (A and B, respectively) and for uCTXII the sum AltmanJSN scores (C and D, respectively) are given as representative figures. Correlation coefficients with p values for all relations are given in table 2. Pearson correlation coefficients with p-values are provided.

Regarding burden, for all radiographic parameters, the baseline radiographic parameter was most strongly related to outcome as can be deduced from the high standardised betas (as compared to the other variables in the models) and the change in explained variance when excluding these variables from the model (i.e. for burden K&L from 0.69 to 0.14). For all three radiographic parameters combining skin pentosidine with uCTXII in the models did not result in significant improvement of predictive ability of the model, supporting the dominant role of the baseline radiographic characteristics. In this analysis both skin pentosidine and uCTXII were predictive of sum K&L score, progression and burden. Interestingly, skin pentosidine did not (significantly) add to prediction of sum Altman JSN, whereas uCTXII did, for progression and burden. On the other hand skin pentosidine added to sum Altman osteophyte score but uCTXII did not, again both for progression and burden. In general BMI did not add to the model with uCTXII as independent biomarker variable but did add to the model with skin pentosidine as biomarker independent variable.

In all cases when progression and burden were expressed as T5-T0 and T5, respectively, instead of AUC, results obtained were very similar (data not shown).
Table 3: Multivariate analysis for skin pentosidine and uCTXII as well as the combination, with radiographic progression and burden as dependent variable.

<table>
<thead>
<tr>
<th>Table 3: Multivariate analysis</th>
<th>Skin pentosidine</th>
<th>uCTXII</th>
<th>combination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>stβ</td>
<td>p</td>
<td>R² (R^2)</td>
</tr>
<tr>
<td>K&amp;L progression AUC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin pentosidine</td>
<td>0.13</td>
<td>0.01</td>
<td>0.07</td>
</tr>
<tr>
<td>uCTXII</td>
<td>NA</td>
<td>NA</td>
<td>0.32</td>
</tr>
<tr>
<td>Age</td>
<td>0.13</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.16</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>K&amp;L burden AUC</td>
<td></td>
<td></td>
<td>0.69 (0.14)</td>
</tr>
<tr>
<td>Skin pentosidine</td>
<td>0.11</td>
<td>0.01</td>
<td>NA</td>
</tr>
<tr>
<td>uCTXII</td>
<td>NA</td>
<td>NA</td>
<td>0.19</td>
</tr>
<tr>
<td>BMI</td>
<td>0.09</td>
<td>0.05</td>
<td>0.76</td>
</tr>
<tr>
<td>Baseline rad.</td>
<td>0.82</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>JSN progression AUC</td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Skin pentosidine</td>
<td>0.15</td>
<td>0.05</td>
<td>NA</td>
</tr>
<tr>
<td>uCTXII</td>
<td>NA</td>
<td>NA</td>
<td>0.76</td>
</tr>
<tr>
<td>BMI</td>
<td>0.13</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>JSN burden AUC</td>
<td></td>
<td></td>
<td>0.87 (0.07)</td>
</tr>
<tr>
<td>Skin pentosidine</td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>uCTXII</td>
<td>NA</td>
<td>NA</td>
<td>0.92</td>
</tr>
<tr>
<td>BMI</td>
<td>0.94</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Baseline rad.</td>
<td>0.25</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Osteophytes progression AUC</td>
<td></td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td>Skin pentosidine</td>
<td>0.24</td>
<td>0.00</td>
<td>NA</td>
</tr>
<tr>
<td>uCTXII</td>
<td>NA</td>
<td>NA</td>
<td>0.22</td>
</tr>
<tr>
<td>BMI</td>
<td>0.33</td>
<td>0.00</td>
<td>0.37</td>
</tr>
<tr>
<td>Baseline rad.</td>
<td>-0.14</td>
<td>0.09</td>
<td>-0.15</td>
</tr>
<tr>
<td>Kidney function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteophytes burden AUC</td>
<td>0.85</td>
<td>(0.08)</td>
<td>0.83 (0.12)</td>
</tr>
<tr>
<td>Skin pentosidine</td>
<td>0.01</td>
<td>0.01</td>
<td>NA</td>
</tr>
<tr>
<td>uCTXII</td>
<td>NA</td>
<td>NA</td>
<td>0.07</td>
</tr>
<tr>
<td>BMI</td>
<td>0.91</td>
<td>0.00</td>
<td>0.91</td>
</tr>
<tr>
<td>Baseline rad.</td>
<td>0.91</td>
<td>0.00</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Standardised betas (stβ) are given, R² is given for the whole model for progression and burden. For burden the R² of the model excluding baseline radiographic data is given additionally (number between brackets). Age, BMI, baseline radiographic characteristics, kidney function, and gender were added as independent covariates, and removed from the model when not contributing.
Discussion

It was studied whether skin pentosidine better, alone or in combination with uCTXII can predict radiographic progression and burden of knee and hip joint damage very early in the osteoarthric disease process. In general skin pentosidine could not improve the predictive ability of uCTXII for progression nor burden of disease. The best predictive models were obtained for burden of radiographic damage, but this was predominantly determined by baseline radiographic damage. Skin pentosidine significantly added to prediction of osteophyte formation whereas uCTXII did not, and uCTXII significantly added to JSN whereas skin pentosidine did not.

Previously we demonstrated in a cross sectional approach that skin pentosidine is higher in participants with more severe radiographic K&L hip and knee joint damage\(^\text{11}\). The present study demonstrates that for progression and burden of disease over 5 years this association is lost. On the other hand, uCTXII at baseline was discriminative for general (K&L) radiographic progression and burden. Interestingly, when osteophyte formation was evaluated as an independent radiographic characteristic, skin pentosidine was related to progression and burden over 5 years follow-up, whereas this is not the case for JSN. But in regression analyses, despite statistical significance, the explained variance was only marginally increased and insufficiently to aid to prediction of radiographic progression in individual patients.

When studying skin pentosidine or uCTXII a different aspect of metabolism is studied. Skin pentosidine can be used to get more insight in cartilage pentosidine levels\(^\text{5}\). The rate of collagen turnover in skin is around 14.8 years, and even longer in cartilage\(^\text{5}\), resulting in accumulation of pentosidine over time into the tissue. When using skin pentosidine in relation to radiographic damage the effect of AGEing on OA during lifetime is investigated. uCTXII level is a marker for cartilage degeneration\(^\text{12}\), at a certain point in time and no cumulative measure as tissue pentosidine levels are. Moreover, pentosidine is studied to be causative in joint degeneration, whereas uCTXII is the result of joint degeneration. Irrespectively, skin pentosidine was compared to uCTXII as uCTXII is at present suggested to be the best predictor of radiographic joint damage in OA\(^\text{12,13}\). In case skin pentosidine would be of use as a predictor, its predictive ability should out reach that of uCTXII. This is of even more importance regarding the invasiveness of taking skin biopsies followed by complex HPLC analysis in comparison to analysing a urine sample by ELISA. This means that in clinical practice uCTXII is much easier to use.

Despite the difference in both markers and the difference in their predictive ability, osteophytes and JSN, respectively, they hardly contributed to each other in predicting radiographic progression. For burden this is reasonably explained by the large predictive ability of baseline radiographic damage, for progression this remains speculative.

For skin pentosidine the strongest relation with JSN was expected since pentosidine formation negatively influences the biochemical and biomechanical aspect of the cartilage tissue\(^\text{25}\). However the strongest relation was observed with osteophyte formation. Osteophytes probably don’t have a direct role in disease progression but may serve as markers of the location and severity of the pathologic process\(^\text{26}\). Cartilage can have focal damage not influencing joint space on radiographs yet. In this early phase osteophyte may already be formed at the areas with focal damage and can be seen earlier on radiographs than JSN.

Synovial inflammation often occurs in OA, in early as well as advanced disease. The synovium can be involved in bone matrix remodelling, synovial macrophages can differentiate to form functional osteoclasts capable of bone remodelling\(^\text{27}\). Inflammatory activity adds to this process\(^\text{28}\). AGEs can activate RAGE and stimulate chondrocytes and synoviocytes in the production of pro-inflammatory cytokines\(^\text{29}\). Osteophytes formation have been related to synovial inflammation\(^\text{30}\). The question is whether AGEs are responsible for
early osteophyte formation through inflammation and so this mechanism can explain the relationship between skin pentosidine and osteophytes progression and burden. In our study we didn’t take diurnal variation into account. Several biomarkers are related to physical activity and food consumption, however this has been suggested not account for CTXII levels\textsuperscript{31}. However, CTXII has a diurnal variation with maximal values at the end of the day\textsuperscript{31}. This means that the time a sample is collected is of importance irrespective of cartilage breakdown. Since we collected our samples at different time-points during the day this can influence the amount of CTXII and so can influence our results\textsuperscript{31}, underestimating the predictive ability of this marker. However, the majority of the samples as collected in the morning where the effect of the diurnal variation regarding uCTXII levels is limited, so we expect that even when all samples had been taken at the same time point the predictive ability of uCTX would not have improved significantly.

For the radiographic burden baseline radiographic damage was clearly the best predictive of all variables far out reaching that of uCTXII or skin pentosidine. When this parameter was excluded the explained variance of the burden of radiographic damage with the other independent variables decreased enormously. For both the hip and knee earlier investigations showed that baseline radiographic parameters are associated with progression of OA\textsuperscript{32,33}. As such this observation fits very well with the present knowledge.

CHECK is considered a cohort of very early OA patients with mostly inconclusive radiographic joint damage at baseline for individual joints (K&L grade \leq 1). Apparently, when taking the sum score of all 4 knee and hip joints, the inconclusive joint damage at an individual level, becomes a strong predictor for overall radiographic OA as assessed by ordinal scales in both K&L and Altman score. Using even more detailed analyses by use of continuous variables of multiple individual radiographic characteristics, by using computer assisted techniques such as knee image digital analyses\textsuperscript{34}, possibly even better prediction models could be provided. Such techniques have been demonstrated to delineate different phenotypes of OA with different tissues and progression rates between phenotypes involved\textsuperscript{35}. It might well be possible that when studying the predictive ability of pentosidine in these different phenotypes, predictive ability would increase for certain of these phenotypes. But clearly the same may be true for uCTXII for other phenotypes.

In conclusion: Skin pentosidine adds to prediction of radiographic progression and burden of osteophyte formation and uCTXII to radiographic progression and burden of JSN, but overall skin pentosidine did not perform better than uCTXII in predicting radiographic damage, and baseline radiographic damage was far out the best predictor of radiographic burden. Neither of both biochemical markers is sufficiently discriminating to aid to prognosis of radiographic progression in individual patients, or performs so consistently that it could function as an outcome in clinical trials.

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In end stage osteoarthritis, cartilage tissue pentosidine levels are inversely related to parameters of cartilage damage.

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AA Polak\textsuperscript{4}
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Osteoarthritis and Cartilage accepted for publication
Abstract

Objectives: Age is the most prominent predisposition for development of osteoarthritis (OA). Age-related changes of articular cartilage are likely to play a role. Advanced glycation endproducts (AGEs) accumulate in cartilage matrix with increasing age and adversely affect the biomechanical properties of the cartilage matrix and influences chondrocyte activity. In clinical studies AGEing of cartilage and its relation to actual cartilage damage can only be measured by surrogate markers (e.g. serum, skin or urine AGE levels and imaging or biochemical markers of cartilage damage). In this study actual cartilage AGE levels were directly related to actual cartilage damage by use of cartilage obtained at joint replacement surgery.

Methods: Cartilage and urine samples were obtained from 69 patients undergoing total knee replacement. Samples were analysed for pentosidine as marker of AGE. Cartilage damage was evaluated macroscopically, histologically, and biochemically.

Results: Cartilage and urine pentosidine both increased with increasing age. The higher the macroscopic, histological, and biochemical cartilage damage the lower the cartilage pentosidine levels were. In multiple regression analysis age is not found to be a confounder.

Conclusion: There is an inverse relation between cartilage AGEs and actual cartilage damage in end stage OA. This is likely due to ongoing (ineffective) increased turnover of cartilage matrix proteins even in end stage disease.
Introduction

Osteoarthritis (OA) is a slowly progressive degenerative joint disease characterized by gradual loss of articular cartilage, of which the aetiology is multifactorial. The incidence of OA increases strongly with age: >50% of the population over 60 years of age shows signs of cartilage damage.

Although age is identified as the main risk factor for the development of OA, the mechanism by which ageing is involved, remains largely unclear. In addition to senescence of chondrocytes, age-related changes in the articular cartilage matrix are expected to play an important role in the susceptibility of cartilage to OA. One of the major age-related changes in articular cartilage is the accumulation of advanced glycation endproducts (AGEs). These AGEs are formed by non-enzymatic glycation, a spontaneous reaction of reducing sugars with proteins. AGEs are irreversible chemical modifications of proteins. High tissue protein turnover results in less accumulation. As such, AGEs accumulate with age in long-lived proteins, for example in collagens and proteoglycans of cartilage tissue. As a consequence of this accumulation of AGEs in cartilage, increased stiffness and brittleness of the tissue occurs, making it more prone to mechanical damage. In addition to affecting the mechanical properties of the cartilage tissue, also cellular processes are disturbed resulting in decreased reparative capacity of the chondrocytes. All together, these effects render the tissue more prone to damage that may eventually lead to the development of OA.

Most of the AGEs are neither yet isolated nor characterized. Therefore, a few well-characterized AGEs are routinely used as marker for the process of non-enzymatic glycation. Pentosidine, a fluorescent AGE, formed by a crosslink between lysine and arginine residues, is often used for this purpose. In post-mortem studies, the cartilage pentosidine amount increases with age. Furthermore, in normal cartilage of joints with macroscopic OA the level of pentosidine is increased compared to cartilage of joints without signs of OA. This suggests involvement of AGEs in the onset of cartilage damage.

In humans, most studies on cartilage AGEs and OA are ex-vivo studies or studies using surrogate markers of cartilage AGEing. Pentosidine of skin biopsies, as a surrogate marker of cartilage pentosidine, weakly correlates with radiographic OA in hips and knees in a cohort with very early OA. Skin fluorescence, as a marker of skin AGEing and with that cartilage AGEing, showed no difference between severe OA versus no OA. Also AGE levels of more easily accessible tissues such as serum and urine are used as surrogates. Serum pentosidine concentrations were significant higher in OA patients compared to healthy controls, however no relation with severity of radiographic OA was found. Others demonstrated that serum pentosidine levels can predict radiographic joint space narrowing after 2 years in knee OA patients fulfilling the ACR criteria and <3 years of symptoms. Urinary pentosidine levels were higher in participants with OA eligible for joint replacement surgery compared to healthy controls. However, urinary pentosidine does not seem to predict cartilage loss on MRI. In a large cohort of early OA no relationship between urinary pentosidine and radiographic OA was found although a relation with urinary CTXII as a marker of cartilage damage was evident.

Clearly, relations between AGEing and cartilage damage are found although results are far from conclusive. This may be due to the limited relation between cartilage AGE levels and AGE levels of surrogate compartments such as skin, serum, and urine. Also surrogate markers of cartilage damage (radiography and MRI) and actual cartilage damage are limited. Thus far, a direct relation between cartilage pentosidine levels and cartilage tissue damage, has not been made. Therefore, the present study compares cartilage AGE levels (as well as urinary AGE levels) in OA directly with actual cartilage damage by use of macroscopic, histological, and biochemical analyses.
Materials and methods

Patients
Sixty-nine patients with severe knee OA, who were eligible for total knee replacement surgery, were included between December 2007 and June 2009. Exclusion criteria were total knee replacement for other reasons than OA and kidney disorders. This study was conducted according to the declaration of Helsinki and received ethics approval. Each patient gave written informed consent before participating in the study. Knee radiographs of all patients were available.

Sample collection
At joint replacement surgery, remaining cartilage was obtained from femoral condyles. This tissue was kept in phosphate buffered saline for less than 4 hours and subsequently processed under laminar flow conditions. The cartilage surface was macroscopically graded: 0=fibrillation or focal degeneration, 1=degeneration at multiple locations, 2=degeneration at multiple locations with focal lesions, and 3=degeneration throughout the tissue with severe focal lesions and focally full cartilage abrasion.

Cartilage was cut aseptically from the underlying bone (full thickness), and cut into small pieces weighing between 5 and 15 mg (accuracy 0.1 mg). Two randomly selected samples were used to determine cartilage pentosidine. Four randomly selected samples of each donor were fixed in 4% phosphate buffered formalin in 2% sucrose and stained with Safranin-O fast green–iron haematoxylin for histochemistry. These samples were graded for microscopic features of OA, using the modified Mankin criteria\textsuperscript{21}. Two observers blinded to the source of the cartilage graded the cartilage and the averages of the two observers and the four samples were taken as representative score of each donor. Twenty samples were randomly selected for biochemistry including proteoglycan synthesis, retention, release, and content.

To obtain a surrogate marker of cartilage pentosidine as well, urine samples of each patient was collected shortly before the operation and stored at -80°C immediately for pentosidine analyses.

AGE measurement in cartilage and urine
Pentosidine was used as a representative measure of AGEs, representing other AGE measures as well\textsuperscript{17}. Pentosidine levels in cartilage were measured as described previously\textsuperscript{17}. 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\textsuperscript{4}. Next, collagen samples were digested for 2 h at 65 °C with 5 units/ml papain (from Papaya latex, Sigma) in 300 ul of papain buffer (50 mM phosphate buffer (pH 6.5), 2 mM L-cysteine, and 2 mM EDTA). An aliquot of the papain digests was subsequently hydrolyzed in 1 ml of 6 M HCl at 100 °C for 18 hours. Hydrolyzed cartilage collagen was dried (Speed Vac; Savant, Holbrook, NY), washed (1 ml milliQ water), dried again, and dissolved in water containing the internal standards pyridoxine (10μM; Sigma) and homoarginine (2,4mM; Sigma). Samples were diluted 4-fold with 1% heptafluorobutyric acid in 10% acetonitril and analyzed for pentosidine content by HPLC\textsuperscript{22}. The column (Varian, Sunnyvale, CA, USA) was equilibrated with 0.15% (v/v) HFBA in 24% (v/v) methanol (solvent A). Elution of the crosslinks and the internal standard pyridoxine was achieved at ambient temperature at a flow-rate of 1.0 ml/min in two isocratic steps: time 0–17 min solvent A; time 17–30 min 0.05% (v/v) HFBA in 40% (v/v) methanol (solvent B). The column was washed with 0.1% (v/v) HFBA in 75% (v/v) acetonitrile (solvent C) for 10 min and equilibrated for 10 min with solvent A, resulting in a total analysis time of 50 min per sample. For amino acid analysis, an
aliquot of the pentosidine samples was diluted 200-fold with 0.1M borate buffer (pH 11.4) and derivatized with 9-fluorenylmethyl chloroformate (6 mM in aceton). After termination of the derivatization reaction and removal of excess reagent by triplicate extraction with pentane, samples were diluted 3-fold with 25% (v/v) acetonitrile in borate buffer (pH 8) and analyzed by HPLC.23

The pentosidine content of collagen samples is expressed as millimoles per mole collagen, assuming 300 hydroxyproline residues per triple-helical collagen molecule.23 A calibration curve (R²=0.999) was used (0 to 12.5 pmol pentosidine/ml). The %CV was on average 6.8% for the various pentosidine concentrations within the calibration curve.

Urine pentosidine was measured by HPLC16 in diluted nonhydrolysed urine (1:1 in 0.025% H₂SO₄) following injection of 50μl on a Whatman partisik 10 SCX column (250*4.6mm). Pentosidine was eluted using an isocratic gradient of 0.025% H₂SO₄ and 100 mM Al₂(SO₄)₃ in 0.025% H₂SO₄. Column temperature was kept 60°C and eluted pentosidine detected by a Jasdo FP-1520 fluorimeter at 328nm (ex) 378nm (em). Purified pentosidine calibrated by mass spectroscopy served as a reference. Urinary creatinine was determined by a standard ELISA (Creatinine assay kit, Cayman Chemicals). Urinary pentosidine values were normalized by urinary creatinine to account for urinary dilution.

Radiographic score
The Knee radiographs were scored by two individuals according to the Kellgren and Lawrence (K&L) score unaware of other data from each patient. The average of both scores was used for statistical analysis.

Biochemical analysis
As measure of the proteoglycan synthesis rate, sulfate incorporation rate was determined over a 4-hour period. After 1 hour of pre-culture (equilibration) culture medium was refreshed and Na₂SO₄ (DuPont NEX-o41-H, carrier free) 370 kBq added as described before. After 4 hour labeling period, the cartilage explants were rinsed once, washed three times for 45 minutes in culture medium under culture conditions, and subsequently cultured, individually for an additional period of 3 days. After this period cartilage and culture medium were separated, cartilage was rinsed in cold PBS and both, cartilage samples and culture medium were snap frozen and stored at -20°C for no longer than 7 days.

For proteoglycan synthesis and –content, cartilage samples were digested in papain buffer as described before. Papain digests were diluted to the appropriate concentrations and glycosaminoglycans (GAGs) were stained, precipitated with Alcian Blue dye solution, and subsequently dissolved in sodium dodecyl sulphate (SDS). The ⁴⁵SO₄²⁻ radioactivity of the sample was measured by liquid scintillation analysis. The ⁴⁵SO₄²⁻ incorporation was normalized to the specific activity of the medium, labeling time and wet weight of the cartilage samples. The proteoglycan synthesis rate is expressed as nanomoles of sulphate incorporated per hour per gram wet weight of the cartilage (nmol/h.g). Blue staining, as a measure for the proteoglycan content, was quantified photometrically by the change in absorbance at 620 nm. Chondroitin sulphate was used as reference. Values were normalized to the wet weight of the cartilage and expressed milligrams of GAGs per gram wet weight of cartilage tissue (mg/g).

Part of the culture medium was used to analyze release of the newly formed proteoglycans and total proteoglycan release. For release of the proteoglycans, GAGs were precipitated from the 3-day culture medium and stained with Alcian Blue. The radio-labeled GAGs were measured by liquid scintillation analysis and normalized to the proteoglycan synthesis rate and expressed as percentage release of newly formed proteoglycans, being a measure of retention of newly formed proteoglycans. For the total release of proteoglycans blue staining
was quantified photometrically by the change in absorbance at 620 nm (chondroitin sulphate used as reference). Values were normalized to the GAG content of the explants and expressed as a percentage release.

**Calculations and statistical analysis**
Because of focal differences in composition and bioactivity of the knee cartilage (specifically in case of severe OA) the results of multiple cartilage samples per parameter per patient (obtained at random and handled individually; 2, 4, and 20 for pentosidine, histochemistry, and biochemistry, respectively) were averaged and taken as a representative value for the cartilage of that patient. Cartilage and urine pentosidine were ln transformed, to obtain normal distribution. Differences between groups were analyzed by Mann Whitney U test, correlations were tested using Pearson or Spearman correlation depending on normal distribution. Multiple regression analysis was used to correct relations for potential confounders, age, gender, and BMI. Data were checked graphically using a histogram and normal probability plot of the residuals. The observed and predicted outcomes were plotted against each other to check for heteroscedasticity. There was a homogeneity of variance and data were normally distributed. \( P \) values less than or equal to 0.05 were considered statistically significant. SPSS software version 15.0 was used.
Results

Baseline characteristics

In Table I the baseline characteristics of the cohort are described. The values for the different parameters are typically for OA patients with severe knee OA who were eligible for total knee replacement\textsuperscript{25}.

Table I: Baseline characteristics of the 69 patients included in this study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
<th>Count</th>
<th>N</th>
</tr>
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<tr>
<td>age (years)</td>
<td>68.8 ± 9.0</td>
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<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>29.1 ± 5.2</td>
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<td></td>
</tr>
<tr>
<td>Ln cartilage pentosidine/collagen (nmol/mol)</td>
<td>1.92 ± 5.2</td>
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<td></td>
</tr>
<tr>
<td>Ln urine pentosidine/creatinine (pmol/µmol)</td>
<td>-0.12 ± 0.51</td>
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<td></td>
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<tr>
<td>histology cartilage</td>
<td>4.78 ± 1.08</td>
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<tr>
<td>PG synthesis (nmol.h.g)</td>
<td>2.33 ± 0.96</td>
<td></td>
<td></td>
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<tr>
<td>PG release (%)</td>
<td>11.69 ± 4.25</td>
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<td></td>
</tr>
<tr>
<td>newly formed GAG released (%)</td>
<td>15.11 ± 8.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG content (mg/g)</td>
<td>24.26 ± 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gender (female/male)</td>
<td>50/19</td>
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</table>

Pentosidine

Cartilage pentosidine levels increased statistically significant with age (R=0.493 <0.001; Fig. 1A). Also the urine pentosidine increased with age (R=0.535, p<0.001 Fig. 1B). Cartilage pentosidine correlated with urine pentosidine; with increasing cartilage pentosidine the urine pentosidine increased (R=0.307, p=0.016; data not shown).

Figure 1: Pentosidine levels with age. The correlation of cartilage (A) and urine (B) pentosidine with age (N=64). For both a positive correlation was found.
Cartilage pentosidine and damage

Cartilage integrity

No clear difference in cartilage pentosidine was found between patients with the highest macroscopic level of cartilage damage (an ordinal scale, macroscopic score >2.5) and those with lower macroscopic damage (macroscopic score ≤2.5) although a tendency can be anticipated relating low cartilage pentosidine levels with high macroscopic damage (Fig. 2A, p=0.192). For the cartilage histology an inverse relation was seen; patients with the highest histological cartilage damage had the lowest cartilage pentosidine levels (continuous scales, R= -0.428, p=0.001; Fig. 2B).

Figure 2: The relation of cartilage pentosidine with the macroscopic cartilage damage (A). The data are presented as mean (square) with 2 times SD (upper and lower limit). When the macroscopic cartilage damage is dichotomized (N=58) no clear relationship between the pentosidine levels and macroscopic score was found although a tendency towards a higher macroscopic OA with lower pentosidine amount can be anticipated. B: Negative correlation between microscopic cartilage damage and cartilage pentosidine levels (R= -0.428, p=0.001; N=59). A higher microscopic OA score correlated with lower cartilage pentosidine levels.

Cartilage biochemistry and chondrocyte activity

The inverse correlation between cartilage damage and cartilage pentosidine levels was further corroborated by the biochemical data. Cartilage with the lowest proteoglycan (PG) content showed the lowest cartilage pentosidine levels (Fig. 3A; although not statistically significant). Proteoglycan synthesis was lowest in cartilage with the lowest pentosidine levels (R=0.443, p<0.001; Fig 3B). The highest release of proteoglycans, resident as well as newly formed, was
found in cartilage with the lowest pentosidine levels (Fig. 2C, R= -0.392, p=0.002 and Fig. 2D, R= -0.400, p=0.002, respectively).

Figure 3: The biochemical markers of the cartilage in relation to cartilage pentosidine (N=60). A: PG content, B: PG synthesis, C: % total PG release, and D: % newly formed PG released (as a measure for retention of newly formed PGs). No correlation is found for cartilage pentosidine with the PG content. The PG synthesis increases with increasing pentosidine, for the % total and % newly formed PGs a negative relation is found.

Pentosidine and K&L score
In general, a high K&L score was found (59/69 ≥ 3; lowest score 2 see Table I). No correlation between the radiographic score and cartilage parameters (biochemical as well as histological; data not shown) were found. The variation in K&L score was low; due to a cohort with severe OA, and therefore a correlation with cartilage parameters is difficult to find. No relationship between cartilage pentosidine levels and radiographic damage was found.

Urine pentosidine and cartilage damage
No clear relations between urine pentosidine and macroscopic and microscopic cartilage damage could be found (data not shown). For the biochemical cartilage parameters, similar correlations were found as for cartilage pentosidine levels, although less profound (PG content: R=0.057, p=0.667; PG synthesis rate: R=0.279, p=0.032; total PG release: R= -0.288, p=0.027; newly formed GAG release: R= -0.292, p=0.025). Also, no relationship between urine pentosidine levels and radiographic damage was found.

Multiple regression analysis
Cartilage pentosidine is strongly correlated with age. Multiple regression analysis was used to separate the effect of age and pentosidine on cartilage damage (Table II). Different outcome parameters were each used as dependent variable and ln skin pentosidine/collagen and age as independent variable entered backwards on either statistical significance or a decrease of 10% or more in the regression coefficient of skin pentosidine. In this multivariate analysis, pentosidine but not age, predict cartilage histology, PG synthesis, % total PG and newly
formed GAG released as independent variable. This implies that cartilage damage is related to cartilage pentosidine without age being a confounder. In most cases the explained variance was around 40% ($R^2$~0.4). This means that cartilage pentosidine explains ~40% of the separate outcome measurements.

If gender and BMI were added in these models no extra explained effect was observed so these factors have no additional effect in explaining cartilage damage (see table IIb in appendix with the additional parameters included).
**Table II: Results of multiple regression analysis** with each of the OA parameters as dependent variable (column 1) and ln skin pentosidine/collagen and age as independent variables entered backwards. The explained variance in each model is shown as $R^2$. The partial regression coefficient for age ($B_{age}$) and ln cartilage pentosidine ($B_{ln\ cart\ pent}$) is given. For cartilage microscopy, PG synthesis, % total PG and % newly formed GAG released the total model shows a significant explanation for the dependent variable, where cartilage pentosidine is the only dependent variable as explanation. P values are indicated and considered statistically significant when below 0.05.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>R square</th>
<th>p</th>
<th>B age</th>
<th>95% CI interval</th>
<th>p</th>
<th>B ln cart pent</th>
<th>95% CI interval</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage macroscopy</td>
<td>0.065</td>
<td>0.154</td>
<td>-0.004</td>
<td>-0.020 - +0.013</td>
<td>0.671</td>
<td>-0.232</td>
<td>-0.560 - +0.095</td>
<td>0.161</td>
</tr>
<tr>
<td>Cartilage histology</td>
<td>0.192</td>
<td>0.003</td>
<td>0.010</td>
<td>-0.024 - +0.043</td>
<td>0.566</td>
<td>-1.103</td>
<td>-1.757 - -0.449</td>
<td>0.001</td>
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<tr>
<td>PG synthesis</td>
<td>0.453</td>
<td>0.001</td>
<td>0.024</td>
<td>-0.005 - +0.053</td>
<td>0.102</td>
<td>0.604</td>
<td>0.028 - +1.182</td>
<td>0.040</td>
</tr>
<tr>
<td>% total PG released</td>
<td>0.392</td>
<td>0.009</td>
<td>-0.003</td>
<td>-0.132 - +0.127</td>
<td>0.969</td>
<td>-3.525</td>
<td>-6.114 - -0.937</td>
<td>0.008</td>
</tr>
<tr>
<td>% newly formed GAG released</td>
<td>0.444</td>
<td>0.002</td>
<td>0.017</td>
<td>-0.238 - +0.272</td>
<td>0.894</td>
<td>-8.270</td>
<td>-13.347 - -3.192</td>
<td>0.002</td>
</tr>
<tr>
<td>PG content</td>
<td>0.231</td>
<td>0.209</td>
<td>-0.059</td>
<td>-0.273 - +0.154</td>
<td>0.581</td>
<td>3.175</td>
<td>-0.544 - +7.974</td>
<td>0.086</td>
</tr>
<tr>
<td>KL score</td>
<td>0.020</td>
<td>0.538</td>
<td>0.010</td>
<td>-0.010 - +0.031</td>
<td>0.322</td>
<td>-0.010</td>
<td>-0.423 - +0.402</td>
<td>0.960</td>
</tr>
</tbody>
</table>
Discussion

The purpose of this study was the direct evaluation of cartilage (and urine) pentosidine levels and the relation with the actual macroscopic, microscopic, and biochemical cartilage damage. Based on literature where surrogate markers of cartilage damage were used, there was a clear positive relation expected (more cartilage damage, higher pentosidine levels). However, the present study, using direct markers of cartilage damage, showed opposite effects for several parameters (more cartilage damage, lower pentosidine levels) in end stage disease.

As is seen in other studies the pentosidine levels increase with age in this cohort (Fig. 1), although we found a lower correlation coefficient compared to the historical data ($R=0.493$ vs. $R=0.93$, respectively). In the latter case, the cartilage samples were obtained from donors of a wider range of age (33-83 compared to 50-88 years in the current study), which might explain the lower correlation coefficient.

We found a lower amount of cartilage pentosidine levels in the cartilage with histological cartilage damage contrasting previous data. This inverse relation was further supported by the correlations with the biochemical data, most clearly in the total PG release and the release of newly formed PGs. A possible explanation for this inverse relation can be the changed turnover of matrix components in OA such as formation of new collagen. It has been demonstrated that collagen AGE levels are greatly influenced by the rate of collagen turnover. In macroscopic normal samples from knees with focal cartilage degeneration the collagen turnover is higher compared to samples from knees without signs of degeneration.

Pentosidine levels in newly formed cartilage tissue (granulated tissue) was lower compared to older cartilage samples in the cartilage obtained from the same joint at joint replacement. Cartilage from OA patients show loss of background AGE fluorescence centered on chondrocytes, and more extensive fluorescence near the surface. The pattern of fluorescence loss is distinct from that of proteoglycan loss, but is generally similar to the distribution of collagen type-II denaturation products. This indicates that chondrocytes in OA cartilage mediate destruction and turnover of AGE-modified collagen, followed by the deposition of new, non-AGE-modified-collagen. Moreover, consistent with the occurrence of increased aggrecan turnover during the OA disease process, aggrecan isolated from OA cartilage contains less pentosidine than aggrecan from normal cartilage. So, in case of more severe OA the cartilage can contain lower amount of pentosidine due to the newly formed collagen not yet modified by the non-enzymatic glycation.

Both OA and AGEs strongly depend on age. In multiple regression analysis we observed that cartilage pentosidine can explain the outcome measures of OA, age not being a confounder (Table II).

Another method to look at the residence time of proteins in tissue is to use the amount of racemization. In nature, amino acids are synthesized as L-isomers. Racemization converts them slowly to the D-form. Aspartic acid is one of the fastest racemizing amino acids. Racemization of aspartic acid can be used to gain information on the residence time of aggrecan in cartilage, the %D-aspartic acid (%D-asp) can be used as a measure of the residence time of a protein. In the present study we found a moderate positive correlation between the %D-asp and pentosidine levels of the cartilage ($R=0.485$, $p<0.001$; data not shown). This indicates that the cartilage with long-lived collagen (higher amount of %D-asp) has a higher amount of pentosidine. For the cartilage histology a negative correlation was found with %D-asp ($R=0.425$, $p=0.001$; data not shown), further supporting that the stronger cartilage damage is associated with increased tissue remodeling and consequently newer proteins in the cartilage tissue. Taken together these results can be an explanation for the inverse relationship with pentosidine and actual OA damage.

It is tempting to evaluate biochemical markers of cartilage turnover to provide additional proof for the enhanced turnover as mechanism of the inverse relationship between cartilage...
pentosidine and OA damage. However, this enhanced turnover may (and will have) occurred over the years of progression of the cartilage damage. Biochemical markers of cartilage turnover would only provide information of turnover at this moment. Racemization of aspartic acid is the result of turnover over the years and as such considered a good support for the proposed explanation.

In this study only pentosidine was measured as a representative measurement of AGEs. Other measurements have been described, i.a. N\textsuperscript{ε}-carboxymethyllysine (CML) and N\textsuperscript{ε}-carboxyethyllysine (CEL). These markers for AGEs were not measured. Because earlier studies showed a close relationship between all these different markers and age\textsuperscript{17}, we assume that other measurements show similar results. Nonetheless, the inclusion of other markers might have strengthen our conclusion.

The negative correlation between the release of total and newly formed PGs with higher amount of pentosidine (Fig. 3B and C) can also be explained due to a changed turnover of PGs or less actual loss from the cartilage matrix. Accumulation of AGEs results in a decreased susceptibility to chondrocyte mediated degradation of the cartilage matrix\textsuperscript{32}. Release of proteoglycans and collagen from the extracellular matrix by both chondrocyte-derived proteinases and synovial fluid proteinases is decreased at increased AGE levels. The exact mechanism by which AGE accumulation results in diminished PG release remains unclear. Accumulation of AGEs may render proteins less susceptible to degradation by interfering with proteinase-substance interactions: AGEs change protein charge and conformation and/or may cause sterical hindrance and thereby interfere with proteinase-substrate interactions. Glycation of cartilage changes biophysical properties of the matrix proteins and thereby possibly affect the permeability of the matrix for proteinases\textsuperscript{33}. In addition, increased inter- and intramolecular AGE crosslinks may diminish the release of degraded cartilage constituents\textsuperscript{34}. Also, production of MMP’s by chondrocytes is decreased in a glycated environment and results in a decreased collagen degradation\textsuperscript{35}. This means that with increased AGE levels less PGs fragments are available for release.

The level of PG content in the cartilage tissue is not statistically significant associated with pentosidine levels. The reason is that proteoglycan content is a less sensitive measurement than synthesis and release and is the cumulative result of changes of synthesis and release over time, whereas synthesis and release itself are momentary (dynamic) parameters. This explains why PG content is not and synthesis and release are related to pentosidine levels.

Urinary pentosidine is measured in a momentum dependent on various factors including peripheral turnover. In these end-stage OA patients we observed the same relations with cartilage turnover as for cartilage pentosidine. In case of early OA we could not find a relation with radiographic OA and urinary pentosidine\textsuperscript{11}. The difference between these observations is that the latter study uses a surrogate marker for cartilage OA (radiographs) and is based on early OA. In case of end-stage OA, as in the present study, the turnover of the cartilage may be more outspoken compared to early OA.

In conclusion, there is an inverse relation between AGEs and actual cartilage damage measured on different levels in end stage OA using the cartilage tissue itself. This is contrasting the existing literature which makes use of indirect measurements. Whether AGEs play a role in the progression of OA remains unclear at this moment.

Acknowledgement
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We thank Paco Welsing for help with the statistical analysis.
**Author contribution**
All authors have made substantial contribution to the study design or acquisition of data, drafting of the article and final approval.

**Conflict of interest**
The authors report no conflicts of interest.
References


### Appendix chapter 8

Table IIb multiple regression analyses with AGE, age, BMI, and gender as independent variables

<table>
<thead>
<tr>
<th>Outcome</th>
<th>R</th>
<th>p</th>
<th>B age</th>
<th>95% CI</th>
<th>B ln</th>
<th>p</th>
<th>95% CI</th>
<th>BMI</th>
<th>p</th>
<th>95% CI</th>
<th>gender</th>
<th>p</th>
<th>95% CI</th>
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<td></td>
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<td>macroscopy</td>
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<td>0.265</td>
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<tr>
<td>Cartilage</td>
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<tr>
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<td>0.011</td>
<td>0.518</td>
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Chapter 9

Summary & Discussion: uncoupling AGE and age?
In human articular cartilage, advanced glycation endproducts (AGEs) are present and increase with age\textsuperscript{1,2}. Pentosidine is one of these AGEs and is frequently used as a marker. It has been demonstrated that age-related accumulation of AGEs affects both biomechanical and biochemical characteristics of tissues, especially those with a low turnover such as articular cartilage. Increased AGE levels of cartilage tissue indeed increases the stiffness and brittleness leading to enhanced susceptibility of articular cartilage to mechanical damage\textsuperscript{3,4}. On biochemical level, accumulation of AGEs was shown to decrease proteoglycan and collagen synthesis by chondrocytes\textsuperscript{5-7}. Based on these data advanced glycation endproducts have been suggested to provide the molecular mechanism by which age-related changes in articular cartilage contribute to the development and the progression of osteoarthritis (OA).

Previous studies have focused on the relationship between AGE and cartilage matrix functioning, chondrocyte activity, as well as on AGEs and progression of OA in vivo (more extensively described in the introduction chapter 1 of this thesis). The studies described in this thesis were focused on the evaluation of the effect of AGEs on the initiation (i.e. development) of OA. The process of AGE formation in vivo, the slow progressive nature of OA development, and the tight relationship of AGE accumulation as well as OA with age, required a combination of animal models and human in vivo and ex vivo studies to gather more insight in this subject.

**Animal studies**

In order to evaluate whether differences in AGE levels – not confounded by concomitant differences in age – affect OA, experimental models are needed. Canine models can be used to study the effect of AGEs in the development and progression of OA. There are several different canine models available for studying the effect of AGEs in OA. First, the canine anterior cruciate ligament transection (ACLT) model for OA\textsuperscript{8}. In this model, the osteoarthritic changes are induced by joint instability as a result of ligament transection, which (since the ligament is not surgically repaired) remains present as a permanent trigger. As such, OA will develop in this model and the progression (rate) of OA can be studied. By selective artificial enhancing AGE levels in articular cartilage of young Beagle dogs, the role of AGE accumulation in the development of OA was studied in the absence of other age-related changes. It was demonstrated that the artificially enhanced AGE levels indeed resulted in an enhanced progression of OA in that model\textsuperscript{9}.

Since the ACLT model uses a permanent trigger for the development of OA only the progression of OA can be studied. A onetime (non-permanent) trigger is more suitable to study the development of OA. For this reason the Groove model was developed\textsuperscript{10}. This model is based on surgically applied damage of the articular cartilage in combination with transient forced loading of the affected joint and shows characteristics mimicking human OA\textsuperscript{11}. Ten and twenty weeks after induction of joint degeneration characteristics of OA are not just the expression of surgically applied damage but are clearly the results of progressive features of (experimental) OA\textsuperscript{12}.
In the Groove model forced loading is applied to the affected joint by fixation of the contralateral leg to the trunk. However, the question whether this forced loading is really necessary in the Groove model and whether this additional loading adds on the development of OA was never studied, but only anticipated on. Therefore, in chapter 2 the role of loading in the Groove model was evaluated. OA was induced in knee joints of beagle dogs according to the Groove model. Forced loading was applied in half of the dogs by fixation the contralateral limb to the trunk, whereas the other group did not receive forced loading. In both groups OA in the joints was evaluated macroscopically, microscopically, and biochemically 20 weeks after induction. For the intensified loading group, the characteristics of OA was statistically significantly more outspoken compared to the group without forced loading, though the differences were small. As such we conclude that loading enhances the development of OA in the canine Groove model. Most importantly, it demonstrated that intensified mechanical loading of a joint (intensity and or frequency) enhances joint degeneration in case primary chondral damage is present.

Previous animal in vivo studies evaluating the role of non-enzymatic glycation were aimed only at the progression of OA. As mentioned, in this thesis we want to investigate the role of non-enzymatic glycation in the development of OA. As it was shown that intensified loading contributes to the development of joint degeneration we designed a study enabling evaluation of the role of AGEs in the development of OA. This was done in two distinct ways. First, we reduced the factor of loading of joints as much as possible but induced OA primary by applying only a few grooves in joints with (AGEd) and without (PBS injected joints) enhanced cartilage glycation (chapter 3). By doing so it was expected that the AGEd joint would develop OA while the PBS injected joint the grooves would (in the absence of substantial loading) be unable to induce OA. However, thirty weeks after surgery, both the AGEd and PBS injected joints show early features of OA. Because the control joints showed already mild features of OA, the attempt to solely study the development of OA was unsuccessful (see also figure above). For both experimental groups, the severity of cartilage damage (proteoglycan content and release) was in-between normal healthy joints and joints with experimental early induced OA according to the original Groove model, when comparing the data in retrospect with those of chapter 2. Features of degeneration were slightly higher in the AGEd joints than in the PBS injected control joints leading to a
progressive increase in severity from controls (retrospect), via minimally grooved/less-loaded controls, to minimally grooved/less-loaded AGEed joints to, damage as observed in the Groove model. This suggests that minor surgically applied cartilage damage with minimal loading induces less severe OA as compared to the Groove model and enhanced AGEs have an additional effect to it. This supports the observation found on progression of OA in the canine ACLT model, but unfortunately did not demonstrate a role of AGEing in development of OA. The second approach in studying the effect of AGE in the development of OA was by severe AGEing of the experimental canine joints without providing a surgically induced trigger for inducing OA. In this set-up the joints were kept fully intact but the trigger to accelerate the development of OA was intensified prolonged loading (chapter 4). After almost 2 years of intensified loading, no induction of OA was achieved in the control group. But disappointingly, a double series of enhancing the cartilage AGE levels to levels multifold of the increase observed in humans over a life span (20 fold), and almost 2 years of intensified loading, no signs of joint degeneration/OA could be found. We concluded that using the current experimental setup, with the proper controls—intensified loading of the joint by exercising the dogs with very 'old' AGEed cartilage is insufficient to induce OA and glycation does not add on to develop OA.

Challenges in studying the effect of AGEs in animal models
Research in animal models remains very important due to the difficulties in studying OA in humans, which stem from poorly sensitive diagnostic tools and the low availability of diseased tissues. When studying the effect of AGEing in OA, animal models can be used to create experimental conditions with varying AGE levels, independent of other age-related changes and directly studying the diseased tissue. Since OA is not only a cartilage disease, but also bone and synovial tissue are effected, the disease can be investigated in all tissues and potential therapeutic targets can also be studied in all tissues involved. In this approach, the use of larger animals has advantages over smaller animals. The development of OA is slower, giving the model more 'human' characteristics. The drawback is that this slow progression also results in a smaller window to induce changes (see chapter 3). But changes that are observed are of more relevance to clinical practice (translational) than those found in fast developing (smaller rodent) models with much higher turnover rates than found in human cartilage. In large animals, the articular cartilage comes closer to that of humans, with respect to the size, structure, and the relatively small number of chondrocytes in an abundant amount of extracellular matrix. The basal cartilage matrix turnover in e.g. dogs in the order of magnitude of that of humans.

To our knowledge the role of AGEing of cartilage, independent of age, has only been studied in dogs. DeGroot showed that artificial enhancement of the cartilage tissue (five-fold, comparable to the 5 fold increase over a human life span), results in an enhanced progression rate of OA. The studies described in this thesis are the first to our knowledge, that attempt to study the effect of AGEs in the development of OA. The ultimate goal of these studies is to develop an animal model that uses a trigger that does not induce OA by itself in healthy 'young' joints, but that does pass a 'point of no return' in case of 'old' artificially AGEed cartilage (figure 2). Only in this way we will be able to demonstrate that AGEing of cartilage during aging, adds to development of OA independent of other age related factors.
Both the ACLT and Groove model are surgically induced OA models. Traumatic OA does occur in humans and mechanical factors are more and more believed to be major inducing factors in development of OA and therefore these models may mimic aspects of the pathogenesis and pathology. One important difference is that humans with a traumatic injury generally decrease use of the affected limb until restabilization has occurred. Animals in the same situation generally do not. Therefore, the disease progression is usually much more rapid in the animal models, thus studying the development of OA may be less amenable.

Both our approaches were unsuccessful to create the experimental conditions in the effect of AGEs on the development of OA could be studied. In the first approach the control group did develop signs of OA and as such we were not studying development of OA, but only progression of OA. In the second approach OA did not develop at all (also not in the AGEd joints). As such it can be concluded that in the first attempt the trigger for inducing OA was too strong and in the second approach too mild. Although we could corroborate a role of AGEing of cartilage in the progression of OA independent of age, we are left with an open end regarding the role of AGEing of cartilage in the development of OA independent of age.

**Human studies**

In parallel to animal models human models were used to investigate the role of AGEs on development of OA. These studies were performed in two separate populations: a very early OA cohort to study the role of AGEing in development of OA, by use of surrogate markers of cartilage AGEing and an end-stage OA cohort to study the actual relation of cartilage AGEing and cartilage degeneration.

One of the biggest challenges of studying OA in humans is defining OA. In the early stage of the disease the clinical symptoms are not very specific. More and more sophisticated methods are used to improve diagnosis including MRI. Irrespective, plain radiography can still be of help in the diagnosis of OA, because of the relative high resolution of radiographs and of course the easily accessibility. Distinct features of OA such as thinning of the cartilage layer represented by joint space narrowing, osteophyte formation, and subchondral sclerosis can easily be detected. Note that radiographs have the advantage that they can be taken weight bearing, providing indirect information on the mechanical quality of the cartilage tissue, this in contrast to MRI data, that in general can only be obtained in non-weight bearing
conditions. The radiographic information can be (semi)quantified by use of several scoring systems. An overall, but widely used, way to score radiographs is the Kellgren and Lawrence score with an ordinal scale of 0-4\textsuperscript{15}. This score summarizes several features of OA with a first focus on osteophyte formation and secondarily joint space narrowing. To evaluate the different components individually, scoring according to the Altman Atlas is used\textsuperscript{16}. In this score the separate features of OA, joint space narrowing, osteophyte formation, and subchondral sclerosis, are semi quantified scored. Also this method uses ordinal scores resulting in more qualitative scoring. The present digital radiographic images also allow evaluation of the different parameters on continuous scales, quantitative scoring. For joint space width narrowing such scores have been described for the knee\textsuperscript{17,18}. For the hip a method was available\textsuperscript{19} to evaluate the joint space width in mm on a continuous scale. We developed a digital method specific for the knee including all different aspects of joint degeneration as observed in the process of OA: Knee Images Digital Analysis (KIDA). In chapter 5 Knee Images Digital Analysis (KIDA) allowing parameters of radiographic OA to be quantitatively measured on continuous scales is described. Minimum joint space width (JSW in mm) is measured as the smallest distance between femur and tibia. Medial JSW and lateral JSW are defined as the mean of four predefined locations in each compartment. The varus angle (in degrees) between the femur and tibia is determined in the frontal plane using the intersection points that determine the bone and cartilage interface; a positive value represents (more) varus and a negative value represents valgus alignment. Height of the lateral and medial tibial eminence are measured in mm. Osteophyte area (in mm\textsuperscript{2}) is determined at the lateral and medial side of both the femur and tibia. Bone density (in mmAl equivalents) is determined at four predefined locations in the lateral and medial femur and tibia, by normalizing the gray values of the subchondral bone region to those of an aluminium reference step wedge that was present on all radiographs. We demonstrated that KIDA is a reliable method to determine small changes in the above parameters on standard standardized radiographs.

To study the very early features of OA in relation to AGEing, the cohort hip & cohort knee (CHECK) was used (chapter 6). CHECK included individuals with pain or stiffness of hips and/or knees related to OA, as such a cohort of individuals with very early signs of OA\textsuperscript{20}. Because in such a cohort it is virtually impossible to obtain data on the level of AGE in the cartilage, surrogate measures had to be studied. In previous studies it has been proposed that skin and urine pentosidine are surrogates for cartilage pentosidine, with pentosidine as a marker for overall AGE. In chapter 6 the amount of skin and urine pentosidine was measured cross-sectionally at baseline in 300 of the participants of the CHECK cohort. We showed that participants with mild radiographic OA had higher skin pentosidine levels compared to participants with no radiographic signs of OA. No difference could be found for urinary pentosidine. Because this comprise very early radiographic OA we also studied a biochemical marker of cartilage breakdown, being urinary CTXII. For participants with mild OA urinary CTXII levels were higher compared to participants with no OA. These higher CTXII levels were associated with higher pentosidine skin levels. Although, the relations were weak, they suggest a possible role for pentosidine in the early phase (development) of joint degeneration, viz. early radiographic OA.

In chapter 6 we investigated the effect of AGEs cross-sectional very early in the disease. In chapter 7 we investigated whether this early in the disease process, skin pentosidine could predict development/progression of OA over a period of 5 years. K&L score as well as Altman atlas scoring of radiographs were used to measure progression of OA (features). In this case urinary CTXII was a comparator, being reported to be one of the best predictors of radiographic progression. It appeared that skin pentosidine related best to osteophyte formation. Surprisingly, because the hypothesis was based on skin pentosidine as a surrogate for cartilage pentosidine, and as such related to susceptibility of cartilage to damage and
limited repair activity. Urine CTXII performed at least as good as a predictor for development/progression of cartilage damage than skin pentosidine did. The combination of both skin pentosidine and uCTXII hardly performed better than the parameters alone. Thus again pentosidine related to OA characteristics very early in the disease (potentially development) although again not conclusive as progression was interfering in this clinical approach.

In most investigations on AGEs in humans surrogate parameters for cartilage pentosidine (i.e. skin, urine or serum pentosidine) and OA (radiography instead of actual cartilage damage) are used. This has clearly limitations. Therefore, in chapter 8 we were able to use cartilage samples for direct evaluation of AGEs and OA in patients undergoing knee replacement surgery. Although this does not provide information on development of OA, it is the first study to evaluate the existence of such a relation. Remarkably, we found an opposite relation of cartilage pentosidine levels and OA severity in this end stage disease: the more cartilage damage the lower the pentosidine levels of the cartilage. This was also opposing previous published data, which use indirect parameters of cartilage damage. A possible explanation for these opposite results is the formation of new collagen, where non-enzymatic glycation has not (yet) taken place. The residence time of the collagen can be measured by the amount of D-aspartic acid. During lifetime the L-isoform of aspartic acid is converted into the D-isoform, so the %D-aspartic acid (%D-asp) can be used as a measurement of the residence time of the collagen. Indeed we found a correlation between %D-asp and pentosidine and negative correlation with the histology of the cartilage. This implies that over the years of cartilage degeneration that lead to end stage OA, a significant number of new collagen molecules have been formed. This observation is unique in a way that it demonstrates that collagen synthesis during disease is less slow as expected to be in healthy cartilage. Moreover it explains the negative relation found between cartilage AGE and damage as observed in this study.

Challenges in studying the role of AGEs in human OA

In general, human studies are most often performed to understand the pathophysiological mechanism of development and progression of OA, in our case the role of AGEs in OA. The ultimate goal is to find treatment modalities for OA. However one encounters several restrictions when studying the disease process in OA in human. The major challenge is the inevitable use of surrogate markers at a biochemical and imaging level. Indirect measurements of biochemical factors potentially involved in the disease as well as the actual cartilage damage (including chondrocyte activity) are used to study the disease process. This is simply because the actual collection of samples of the cartilage tissue (and other joint tissues) may interfere with the disease process and as such are considered in generally unethical to obtain.

In the present thesis radiography was used as an imaging marker of actual joint damage in addition to (in some studies) biochemical markers of tissue turnover. Biomarkers are molecules or molecular fragments that are released into biological fluids from extracellular matrix turnover and/or cellular metabolism of articular cartilage, subchondral bone and synovial tissue. Unfortunately, none of the current biochemical markers is sufficiently discriminating to aid to diagnosis and prognosis of OA in individual or limited numbers of patients, or performs so consistently that it could function as an outcome in clinical trials. As such our work was restricted to uCTXII, which is at present considered to be the best performing biochemical marker.

The second part of this thesis was also primarily performed to evaluate the role of AGEs in development of OA. In the very early OA cohort CHECK pentosidine seemed to relate to the presence of OA features (chapter 6) as well as development of early OA features (chapter 7). But the fact that OA features were already present and as such pentosidine related also to
Discussion

burden of disease over 5 years follow-up (chapter 7), demonstrates clearly that progression and development cannot fully be untwined in clinical studies. This was the reason in the first place that also animal in vivo studies were performed in parallel.

In this thesis the primary question was: are AGEs responsible for the development of OA. Unfortunately, we are unable to answer this question at the end of this dissertation. We showed that AGEs have a limited role in the progression of OA in animal experiments (chapter 2&3) and human studies (chapter 6&7). For the development of OA no clear undisputable evidence was found (chapter 4&6). This is either due to the fact that no relationship exists or that the models used were still not sufficient enough. But apparently the role for cartilage AGEing in development of OA is not a strong one.

Major disadvantages of the above-described studies are: the heterogeneity of OA and the fact that OA is a whole organ disease rather than an isolated cartilage disease. Both may be of influence in the ‘negative’ outcome of our studies.

Subsets of OA

Osteoarthritis can develop through different pathophysiological mechanisms, ie traumatic, metabolic, aging, and genetic. In case of studying AGEs the aging mechanism would be the most interesting one. However, finding a cohort with participants solely having OA due to age is impossible. In a major part of the cohort additional acquired and inherited condition will be present and involved in the OA process. It would be of interest in future studies with potentially newly developed techniques (markers) to isolate specific subpopulations of the disease in which AGEing is causative in development of OA. This phenotyping of OA is a rapid growing area of interest and might result in breakthroughs also regarding the role of tissue-AGEing, although clear results have still to be awaited.

OA disease of whole organ

OA is not merely a disease of the cartilage tissue; also bone, synovial tissue, and soft tissues such as ligaments and muscles have a role. Synovial tissue inflammation happens in early as well as late phases of the disease but is clearly less outspoken compared to synovitis in rheumatoid arthritis. Also changes in subchondral bone are seen, such as subchondral sclerosis and osteophyte formation. Several of these bone changes take place not only during the final stage of the disease, but also at the onset of the disease, possibly (sometimes) before cartilage degradation. This finding led to the suggestion that subchondral bone could initiate cartilage damage (at least in specific subpopulations).

Also the surrounding tissues, i.e. ligaments and muscles, are of importance in the development of OA. If the surrounding tissues fail, the loads placed on the joint tissues are abnormal distributed to the joints leading to abnormal loading and causing OA.

AGEs do not only accumulate in cartilage tissue, but also in tendons, ligaments, synovial membrane, and subchondral bone. Age-related accumulation of AGEs has been observed in human bone, resulting in increased brittleness. Tendons and ligaments in joints become glycated with advancing age, which likely results in increased stiffness and therefore increased susceptibility to damage.

Ideally, studying the effect of AGEs on the development of OA would consider the whole organ. In animal studies the focus is on the cartilage tissue and changes in the synovial tissue. Bone structure and other surrounding tissues are hardly measured, although possible. In human studies, when using radiographs as a measurement for OA, mainly cartilage and bone parameters can be used, leaving the effect of ligaments aside as well as synovial inflammation. Ideally studying OA should involve all joint tissues, however the measurement of all tissues involved is difficult.
**Future directions**

For the future studies other approaches in animal experiments are required. The use of spontaneous animal models of OA may not be very useful; all animals will develop OA so the development of OA cannot be studied. Genetic knockout mice can be used, although this is a small animal with its inherent disadvantages (e.g. rapid tissue turnover, high cartilage cellularity). It can be assumed that AGEs are responsible in specific circumstances. In case of knocking out specific (additional) pathways, OA may develop in these mice only when artificial enhancement of AGEs is used. Also, the induction of chondral defects on non-weight bearing areas may be used. In horses chondral defects in non-weight bearing areas can be repaired\(^{30}\). When artificial enhancement of AGEs is performed in this situation the question is whether repair is still possible, or whether a point of no return is crossed. When a longer follow-up period is used probably OA can develop in the AGEd and not in the control joints.

Studying OA in humans would ideally be done in a better defined groups of patients, as suggested above. Stratification of patients cohorts into subgroups based on biochemical markers, genotype, lifestyle etc would reduce heterogeneity in study populations, and consequently increase power to identify relevant processes and changes. For the current research question, selecting OA patients with a certain level of e.g. insulin resistance could be relevant.

However, we should also keep in mind that apparently, despite all *in vitro* evidence of impaired biomechanics and impaired repair capacity of human cartilage tissue as a result of artificial AGEing, AGEs in a lifetime physiological range are not, at least not a major, cause of initiation of joint degeneration, but only add to progression of damage. In this respect it is worthwhile to notice that in all *in vitro* experiments performed thus far, artificial AGEing exceeded the levels that can be obtained in a natural life span of humans.

**Overall conclusion**

The overall conclusion of this thesis is that Ch2) loading is involved in progression of OA; Ch3) *in vivo* AGEs can enhance the progression of OA (thus corroborating earlier studies), Ch4) but that development of OA in a canine model could not be demonstrated unambiguously. Ch5) KIDA allows detailed quantification of radiographic characteristics of OA. Ch6) In humans in very early OA higher pentosidine relates to the presence of OA, Ch7) as well as progression of OA. Ch8) In end stage disease, new cartilage tissue is formed and so an inverse relation of cartilage tissue pentosidine with severity of OA is present. Ch9) We still expect that AGEs do play a role in OA, however, regarding development of OA it is hard to proof, and the causality is definitely not a strong one.
Discussion

References


