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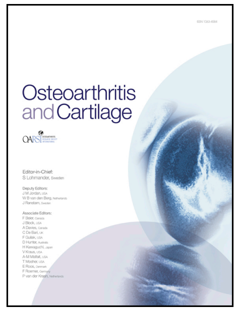

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Potential diagnostic value of a type X collagen neo-epitope biomarker for knee osteoarthritis

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Running title: A neo-epitope of type X collagen as a biomarker

Abstract

Objective: Phenotypic changes of chondrocytes toward hypertrophy might be fundamental in the pathogenesis of OA, of which type X collagen (Col10) is a well-known marker. The purpose was to develop a specific immunoassay for blood quantification of a newly identified neo-epitope of type X collagen to assess its diagnostic value for radiographic knee osteoarthritis (OA).

Methods: A neo-epitope of Col10 was identified in urine samples from OA patients. A monoclonal antibody against the neo-epitope was produced in Balb/C mice. The enzyme responsible for the cleavage was identified. Immunohistochemical detection of this neo-epitope was performed on human OA cartilage. An immunoassay (Col10neo) was developed and quantified in two clinical studies: the C4Pain-003 and the NYU OA progression study. ROC curve analysis was carried out to evaluate the discriminative power of Col10neo between OA and RA.

Results: A neo-epitope specific mAb was produced. The Cathepsin K-generated neo-epitope was localized to the pericellular matrix of chondrocytes, while its presence was extended and more prominent in superficial fibrillation in the cartilage with advanced degradation. In the C4Pain study, a higher level of Col10neo was seen in subjects with greater KL grade. The group of the highest tertile of Col10neo included more subjects with KL3-4. In the NYU study, Col10neo was statistically higher in OA than control or RA. ROC curve analysis revealed area under the curve was 0.88 (95% CI 0.81-0.94).

Conclusion: Our findings indicate that Col10neo linked to hypertrophic chondrocytes could be used as a diagnostic biochemical marker for knee OA.

Key words: chondrocyte hypertrophy, assay, biomarker, type X collagen, Osteoarthritis, Cartilage

Introduction

Osteoarthritis (OA) is the most common joint disease, which is characterized by cartilage damage and impaired joint function[1]. It can implicate synovial joints, but knees, hands, hips, and spine are usually affected by this disease[2]. A range of risk factors, such as aging, gender, obesity, early trauma, and heredity are associated with osteoarthritis of the knee[3].

Although substantial progress has been made to understand the mechanisms leading to degradation of the cartilage matrix in OA, the exact pathogenesis of OA still needs to be elucidated. Supported by emerging evidence hypertrophy of chondrocytes, the only cells present in cartilage is a key event in the development of OA[4, 5,6,7,8]. The time course of expression of type X collagen, a specific marker of hypertrophic chondrocytes, and MMP13 has shown to be significantly induced during OA progression in a surgically induced joint instability OA model[9,5]. In sections of human osteoarthritic cartilage, type X collagen was found around hypertrophic chondrocyte clusters in the deep zone close to tidemark[10]. Also, Eerola et al. demonstrated that intense immunostaining of type X collagen was observed specifically at sites of osteophyte formation and surface fibrillation[11]. Furthermore, the mineralization process has been shown in OA cartilage indicating that articular chondrocytes from OA joints develop terminal differentiation similar to those in the growth plate during endochondral ossification[12]. Conclusively, chondrocyte hypertrophy is associated with OA.

Cartilage degradation is a common feature for rheumatoid arthritis (RA), a systemic autoimmune disorder as well. The degradation of cartilage matrix in both OA and RA is mediated by the overexpression of enzymes, mostly of MMPs and ADAMTSs[13,14]. However, the driving force behind the development of these two disorders is different as summarized by Pap et al. in a review[15]. In OA, cartilage loss occurs as part of a phenotypic shifting of chondrocytes, while in RA, cartilage damage is considered a result of activated synovial fibroblast-like synoviocytes (FLS) and monocytes.

In the current study, we have discovered a neoepitope of type X collagen, ^{479}GIATKG, in the urine samples of osteoarthritis patients. The Cathepsin K-mediated neo-epitope is in the region near the carboxyl-terminal end of the triple helix. We produced a neo-epitope specific monoclonal antibody and identified the productive enzyme responsible for the generation of the neo-epitope *in vitro*. We performed immunostaining of osteoarthritis cartilage to show the distribution of the neo-epitope. We developed an enzyme-linked immunosorbent assay (ELISA). In two clinical studies, blood levels of type X collagen degradation biomarker-Col10neo are associated with Kellgren-Lawrence (KL) grade radiographic severity. Most importantly, Col10neo biomarker could distinguish between OA and RA and suggest the potential diagnostic value of this biomarker for knee OA.

Methods

Materials

Unless otherwise stated, all materials used for experiments were ordered from Sigma-Aldrich (Copenhagen, Denmark) or VWR (Rodovre, Denmark). The synthetic peptides were purchased from GenScript (USA).

Selection of the sequence for immunization

Ten urine samples of peri/postmenopausal women with knee pain undergoing arthroscopy were analyzed by GC/MS and the Mascot database was searched for type X collagen neo-epitopes. Seventeen peptide sequences were found to be unique to human type X collagen. Two sequences carrying the same free c-terminus located at amino acid (aa) position 478' (Accession No.: Q03692; UniProt) were discovered in the urine from OA patients, indicating the cleavage occurring between the bond of A^{478}-^{479}G. The first 10 aa, ^{479}GIATKGLNGP^{488}, of the free N-terminal end generated by this cleavage was selected for immunization. Further, a sequence alignment with different species was conducted using the Basic Local Alignment Search Tool (BLAST).

Monoclonal antibody (mAb) production

Six female Balb/C mice of 6-7 weeks of age were used for monoclonal antibody production and the experiment was approved by the Danish Animal experimentation Council. Six mice were housed in the same cage (the standard type III cage, Scanbur) in a ventilated closet and provided with free access to tap water and food. The 12-hour light/dark cycle was used in the animal facility with a temperature of 18-22^{0}C. Wood shavings were used for bedding. The mice were immunized subcutaneously with emulsified GIATKGLNGP-GGC-KLH with Sigma Adjuvant System®(S6322, Sigma-Aldrich). 100µg of emulsified KLH-conjugate with adjuvant was repeatedly injected into mouse every 3rd week until stable titer levels were obtained. The behavioral assessments were carried out throughout the study. Red bumps after injection were expected and observed. There was no skin infection observed at the sites of injection.

The mice with the highest antibody titer and best reactivity towards the specific peptide, GIATKGLNGP, were chosen for fusion and boosted intraperitoneally (i.p.) with 100 µg of 100 µL KLH-conjugate three days before fusion. The isolated splenocytes were fused with murine myeloma cells, SP2/0-Ag14 (ATCC®CRL-1581™). Hybridoma cells were selected by using HAT (hypoxanthine-aminopterin-thymidine) medium. Supernatants were screened against GIATKGLNGP-k (Biotin) Limiting dilution method was used to get monoclonal antibody and isotype was tested with kit (5300-05, Southern Biotech). Hi Trap Protein G column (17-0404-01, GE healthcare) was used for purification.

^{479}GIATKG specific immuno-assay (Col10neo)

A purified monoclonal antibody was first tested for specificity against three synthetic peptides 1) specific peptide (GIATKGLNGP), 2) elongation of the specific peptide (AGIATKGLNGP), 3) truncation of the specific peptide (IATKGLNGP), and two truncated recombinant type X collagen proteins [Col10(AA 479-680) and Col10 (AA 450-680)] respectively in a competitive immunoassay. The optimized competitive immunoassay protocol: a 96-well streptavidin pre-coated microplate was coated with 1ng/mlGIATKGLNGP-k(Biotin) dissolved in 50mM PBS-BTB for 30 min at 20^{0}C. Then, the plate was washed five times with wash buffer (20 mM Tris, 50 mM NaCl, pH 7.2). 20µL of standards,controls or samples were added to appropriate wells, followed by 100µL of 23ng/ml 2F4in 50mM PBS-BTB buffercontaining 5% Liquid II (Roche Diagnostics, Germany), and incubated overnight (20±1 hr) at 4^{0}C. After five times wash, 100µL of secondary antibody (115-035-003, Jackson ImmunoResearch) was added and incubated for 1hr at 20^{0}C. The plate was washed five times again. Finally, 100µL 3,3',5,5'-tetramethylbenzidine (TMB) was added and incubated for 15 min at 20^{0}C in the dark. The colorimetric reaction was stopped by adding 100µL stopping solution (1% H_2SO_4) and measured at 450nm with 650nm as the reference.

The inter- and intra-plate variation were determined by ten independent runs of the quality control panel in duplicate. The lower limit of detection (LLOD) was calculated as 3SD of the mean value of 21 zero standards. The optimal range for quantitative measurements is defined as the boundary between the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). Linearity of the assay was evaluated.

In vitro cleavage of human cartilage and western blot

Articular cartilage biopsies from OA patients who underwent knee replacement surgery were obtained from Gentofte Hospital (Gentofte, Denmark). The collection and retrieval of the biospecimens complied with international ethical guidelines for handling human samples and patient information. All participants signed informed consent, and the study was approved by Danish authority. To identify the enzymes responsible for the cleavage of the A^{478}-^{479}G bond in Col10, a knee cartilage pool of 10 OA patients undergoing knee replacement surgery was cleaved by numerous proteases including matrix metalloproteases-2, -9, -13 Cathepsins K, B, S, and A disintegrin and metalloprotease domains with thrombospondins motifs-4, -5. Briefly, pulverized cartilage sample (30mg) was incubated with 1µg each enzyme in a 0.5ml Eppendorf with 250µL digestion buffer as previously reported[16]. The digestion was carried out for 24h in replicates. The reaction was quenched by adding EDTA (5mM) or E64 (5mM).

Cleaved products from Cathepsin K and control were suspended in the gel-loading buffer and electrophoresed on 4-12% Tris-Glycine gradient gel and transferred to nitrocellulose by iBlot®. The blots were blocked with 5% BSA in TBST, washed and probed with antibodies of 2F4 or 11G8 (antibody against the carboxyl end of type X collagen developed in our previous study)[17], in the absence or presence of blocking peptides in parallel.

Immunohistochemistry (IHC)

Cartilage with bone specimens for IHC study was performed in collaboration with Frederikshavn Hospital (Denmark) approved by Danish authority (N-20110031). All participants signed informed consent. The specimens isolated from OA patients who underwent knee replacement surgery were fixed and decalcified, and then embedded in paraffin. Cartilage sections (5μm) were deparaffinized and hydrated, followed by antigen retrieval using Pronase E (10165921001, Roche) at 37^0C for 10 min. The sections were treated with 0.5% casein in Tris Buffered Saline (TBS) for 30min at room temperature to block unspecific binding. Immunostaining was performed using antibody 2F4 or 11G8. Non-sense mouse IgG was used as a negative control (X0931, Dako, Denmark). Immunoreactivity was visualized with peroxidase-labeled anti-Mouse and diaminobenzidine (DAB, Dako, Denmark). Counterstaining was with Mayer's hematoxylin. The micrographs were taken using an Olympus microscope BX60 equipped with an Olympus C5050 digital camera.

Study participants

Plasma samples were retrieved from two cohorts, the C4Pain study approved by the local ethics committee (N-20100094)[18] and the NYU OA progression study by the institutional review board (IRB) of NYU School of Medicine [19,20]. All participants provided informed consent before enrollment.

Briefly, the C4Pain is a cross-sectional study. It comprised 281 individuals with radiographic KL grade 0-4. Since most patients had bilateral knee OA, the knee with the maximal pain intensity for the last 24 hours was selected as the signal knee. Sufficient samples were available only from 253 enrollees and categorized into four groups based on the KL grade since the radiographic features instead of pain were the primary focus of the current study.

The NYU study comprised 21 non-OA healthy controls (KL≤1 and no pain in either knee), 146 symptomatic knee OA patients and 36 RA patients at baseline. These 146 OA patients were further followed up for 24 months. The inclusion and exclusion criteria, radiographic assessments were taken at baseline and 24 months and plasma were collected at baseline as described previously[19,20]. Plasma samples were available from 20 non-OA healthy controls, 142 OA subjects and 34 RA subjects at baseline. In the present study, we only investigated the level of Col10neo in different groups at baseline.

Statistics

Data were analyzed using GraphPad Prism 6 or MedCalc 16.8. For normality check, a Shapiro-Wilk test for all variables of interest was performed. Between-group comparison for age, BMI and VAS score, the one-way ANOVA with *post-hoc* Tukey-Kramer test was used. Plasma Col10neo data were logarithmic transformed in all analyses. The ROC curve analysis was performed. The area under the curve was used to determine how well Col10neo can distinguish between OA and RA groups. A P value<0.05 was considered statistically significant. One asterisk (*) if p<0.05; two (**) if p<0.01; three (***) if p<0.001 and four (****) if p<0.0001.

Results

Sequence alignment of the selected peptide across species

Two fragments sharing the identical C-terminal end, ^{463}PGSKGDPGSPGPPGPA^{478} and ^{465}SKGDPGSPGPPGPA^{478} were identified by mass spectrometry in the urine samples of OA patients (Fig. 1A), indicating the presence of a cleavage site existing between A^{478}-^{479}G. A 10aa length peptide from the free N-terminus generated by the cleavage, ^{479}GIATKGLNGP, has been chosen for immunization. The blast shows the sequence is unique to human type X collagen. Sequence similarity across species shows 100% identity between human and mouse, while a mismatched aa contained in rat or bovine compared to human sequence.

Technical performance of the Col10neo assay

A monoclonal antibody 2F4 (isotype: IgG2b, κ) targeting the neo-epitope, ^{479}GIATKG, was produced from a hybridoma and purified by HiTrap Protein G affinity column. To test the specificity, the reactivity of 2F4 toward a biotinylated synthetic peptide, GIATKGLNGP-k(Biotin), completely displaced by adding of 500nM selective peptide or equivalent amount of truncated recombinant type X collagen with free ^{479}Glycine (AA 479-680). In contrast, a slight or no displacement of binding between the immobilized specific peptide and 2F4 was observed either with elongation/truncation of the specific peptide or cleavage-spanning recombinant type X collagen (AA 450-680) at the same concentration (Fig. 1B). This indicated that 2F4 was specific to the neo-epitope instead of intact type X collagen.

. The technical performance of this assay is summarized as follows: IC50 of 41.9ng/ml, inter-, intra CV% of 4 and 9.7%,. The detection range was 15-426ng/mL. 8-64 fold for EDTA-plasma and 4-32 fold for serum samples are of high dilution linearity. (See supplementary table S1).

Cathepsin K-derived ^{479}GIATKG

To investigate the responsible enzyme for cleaving Col10 A^{478}-^{479}G bond, various proteases at similar concentration and incubation time were incubated with human cartilage and evaluated their relative efficiency to generate the neo-epitope of ^{479}GIATKGLNGP. We did not identify release of Col10neo in all tested MMPs or ADAMTSs compared to untreated control. However, among the proteases, Cathepsin K yielded substantial amount of Col10neo release (Fig. 2A). In an immunoblot, major band detected by 2F4 mAb in Cathepsin K degraded cartilage was ~50kDa representing the trimeric form of the NC1 domain. In a completion Western blot, GIATKGLNGP peptide blocked 2F4 mAb recognition of fragment carrying the neo-epitope of ^{479}GIATKGLNGP. Furthermore, mAb 11G8 detected a 18kDa protein band was which is most likely the monomeric NC1 domain of type X collagen (Fig. 2B).

Immunolocalization of ^{479}GIATKG in cartilage

To further define the distribution of ^{479}GIATKG, serial sections of articular cartilage from 3 TKR (total knee replacement) patients were stained with mAb 2F4 (Col10neo) and 11G8 (anti-C terminus of type X collagen) and non-sense mouse IgG of cartilage. A representative histological images of damaged and undamaged cartilage biopsies 1) the normal-arranged chondrocytes, smooth surface and mild degradation of superficial zone proteoglycan (Fig. 3A), 2) loss of smooth surface and moderate loss of proteoglycan (according to Mankin grading system[21]) (Fig. 3B) and 3) chondrocyte clusters, fibrillated surface, cleft and severe reduction in safranin O/Fast green (Fig. 3C) were used for general assessment of degree of loss of proteoglycan, chondrocyte morphology, and Col10neo expression. In mild and moderate degradation of cartilage, GIATKG neo-epitope was localized to the peri-cellular matrix of chondrocytes, while in severe OA its presence was extended to the territorial matrix of chondrocyte clusters and more prominent in superficial fibrillation (Fig. 3D-F). The similar staining pattern was observed with C-terminus 11G8 mAb (Fig. 3G-I).. We did not observe any staining with non-sense mouse IgG (Fig. 3J-L).

Association between KL grade and plasma Col10neo levels in the C4Pain study

The demographic characteristics of these four groups based on radiogrpahic KL score are summarized in table 1. Because the mean age of the participants was >60 years, few with a KL 0 and those with a KL of 1 were classified into the same group. 57% of the subjects involved in this study were with a KL of 2 and assigned to group 2. There was no significant difference in gender distribution within each group. The mean age of KL 4 group was significantly higher than KL 0-1 group (p< 0.005). There was a clear, but not significant trend of increased BMI with increased radiographic grades.. There was a relationship between the VAS score and K/L grade, but did not reach statistical significance.

The mean ± 95CI% concentrations of Col10neo for in each KL groups: KL 0-1 2.6 [2.316-2.884] µg/mL, KL2 3.288[2.885-3.691] µg/mL, KL3 3.435[2.729-4.141] µg/mL and KL4 3.517[2.599-4.435] µg/mL, respectively. Although increased Col10neo levels associated with a higher KL grade, but it was not statistically significant (Fig. 4A). However, when subjects were divided into tertiles based on Col10neo levels, we found that the number of subjects with a KL 3-4 was greatest in the highest tertile of the Col10neo group (Fig. 4B).

Plasma Col10neo levels in the NYU study

The result from the C4Pain study encouraged us to investigate the potential use of Col10neo as a diagnostic biomarker in another independent cohort of control and OA subjects. We utilized NYU Progression study which consists of non-OA healthy control, OA, and RA. In this cohort, percentage of females was higher in OA and RA groups than in the healthy control group. The mean age was significantly different in control and OA groups (P<0.05). However, the subjects were significantly younger in RA than in the OA group (Table 2; p<0.0001). There was no significant difference in BMI across groups.

The mean ± 95CI% concentrations of Col10neo in control, OA and RA groups were 2.95 [2.71-3.19] µg/mL, 4.04 [3.84-4.25] µg/mL and 2.55 [2.29-2.81] µg/mL, respectively (Fig. 5A). Plasma Col10neo level was significantly higher in OA than healthy control (p=0.0002) and RA (p<0.0001). Tests of Between-Subjects Effects have shown that there was an overall statistically significant difference in Col10neo levels between different groups when adjusted for covariates (p<0.001). After adjusting for covariates -age, gender, and BMI, Col10neo level remained significantly higher in the knee OA patients compared to other two groups (Table 3).

ROC analysis was carried out to evaluate the discriminative power of Col10neo between OA and RA. The area under the ROC curve (AUC) was 0.875 [0.806-0.943] (P<0.0001), indicating there is good separation of Col10neo between the two groups (Fig. 5B).

Discussion

Our current findings suggest that the Cathepsin K-generated neo-epitope of type X collagen, ^{479}GIATKGLNGP can be detected in blood circulation, where its concentration reflects the hypertrophic change in phenotype of chondrocytes in articular cartilage and shows good separation of OA from RA.

Therefore, this neo-epitope can serve as a biomarker for hypertrophic chondrocytes and may have many potential clinical applications.

Type X collagen is a short and non-fibril forming collagen containing three identical chains. Type X collagen is well-recognized as a specific marker for hypertrophic chondrocytes[22]. It is synthesized primarily by hypertrophic chondrocytes in the growth plate of fetal cartilage and believed to regulate bone mineralization during endochondral ossification[23]. The evidence for the up-regulation of type X collagen in osteoarthritic cartilage on mRNA level or protein level by immunohistochemistry has been found in multiple studies[10,24,22,25,26]. In the current study, we have observed that type X collagen was stained pericellularly with antibodies to the neo-epitope or C-terminus of type X collagen and distributed extracellularly around chondrocyte clusters in the fibrillated superficial layer of osteoarthritic cartilage. This is consistent with previous studies regarding the deposition of type X collagen by chondrocytes in OA cartilage[10,22]. Our findings confirm the notion of chondrocyte phenotype modulation in OA that chondrocytes in diseased cartilage undergo terminal differentiation to hypertrophy. Moreover, some characteristics of OA such as remodeling of extracellular matrix by proteases, vascularization and focal calcification of joint resemble the physiological differentiation process of chondrocyte during skeletal development[4,27,7,12,6].

The *in vitro* cleavage of human cartilage suggests that the neo-epitope of type X collagen, ^{479}GIATKGLNGP, was efficiently generated by Cathepsin K among a series of tested proteases. Collagenase is known to be capable of cleaving type X collagen at least two cleavage sites, G^{151}-^{152}I and G^{479}-^{480}I, within its triple-helical domain[28,29]. One site is near the amino-terminus of the triple helix, and another site is close to the carboxyl terminus of the triple helix. As a consequence, these two cleavages generate three products, a 32-kDa fragment containing the major triple-helix domain, a 9-kDa fragment from the amine-end of type X collagen and another 18-kDa fragment containing the entire globular domain at the carboxyl end. Interestingly, the newly identified cleavage site from human OA urine in the current study was verified to be generated by Cathepsin K. This cleavage site at A^{478}-^{479}G bond is located an amino acid apart from the proposed collagenase G^{479}-^{480}I. Not surprisingly, both bonds are in the same region where the Gly-Ile-Ala triplet was disrupted by two additional amino acids from the next triplet. Because of the interruption, the region is inevitably susceptible to attacks by a protease. Furthermore, increased expression and activation of Cathepsin K has been reported in a transgenic mouse model for osteoarthritis and human osteoarthritic cartilage[30,31]. It is still uncertain whether cleavage of collagenases occurs prior to cleavage of Cathepsin K. Further investigation is required. To sum up, our data suggest that Cathepsin K is potentially involved in the turnover of type X collagen which is expressed by hypertrophic chondrocytes in OA.

Most interestingly, a recently published study has shown that a group of fragments containing the entire C1q domain and variable portions of the attached linker and collagenous region designated as CXM, was a degradation product of type X collagen during endochondral ossification and an ideal biomarker for the overall growth plate activity[32]. The serum-based assay for CXM used in this study was built upon a monoclonal antibody X34 specifically targeting a multimeric form of the NC1 domain, the ~50kDa NC1 region[10]. In the present study, a similar major band of 50 kDa was detected in the Cathepsin K-generated human cartilage products by 2F4, a mAb specific to the neo-epitope ^{479}GIATKGLNGP. It most likely consists of the compact coiled C1q-like domain which has been reported to resist further proteolysis in other studies[33,34]. In our previous study, 11G8, an antibody raised against the last 10 amino acids at the carboxyl end of type X collagen only recognizes the monomeric NC1 domain, ~18kDa [17]. Given that the dominant form of the NC1 domain in circulation is an intact trimer, it is not surprising that Col10neo levels are generally higher than C-Col10 in the same samples of clinical study C4Pain.

To the best of knowledge, it is the first time that a single biomarker has shown the possibility of distinguishing OA from RA. Although both arthritis diseases affect joint, the molecular mechanisms of cartilage breakdown are quite different in OA and RA. The role of terminally differentiated hypertrophic chondrocytes in OA is still controversial. One study has shown the loss of expression of SOX-9, a transcription factor inhibiting the differentiation progression of chondrocytes during endochondral ossification, but a significant down-regulation of type X collagen gene expression in chondrocytes from OA patients compared to age-matched controls[35]. However, there is increasing evidence to support changes in the phenotypic state of chondrocytes in OA. One important study demonstrated that the chemokines were retained by binding to heparan sulfate proteoglycans to maintain chondrocyte resting phenotype. The release of chemokines during cartilage degradation in OA contributes to chondrocyte phenotype shift[36]. Further damage is caused by proteolytic enzymes secreted by hypertrophic chondrocytes which in return will lead to more loss of stability of chondrocytes, amplifying a vicious circle. To explain the discrepancy between studies, there might be different patient subgroups existing as shown by the scattered Col10neo levels in the OA group in the current study. The question if chondrocyte hypertrophy differentiation is a generalized phenomenon in OA needs to be further investigated. RA is a chronic systemic inflammatory disease instead, which is characterized by synovial lining cell proliferation, inflammatory cell infiltration, and destruction of cartilage and bone. Therefore, the underlying molecular mechanisms behind the pathogenesis of OA and RA may be different. We speculate that in OA, the hypertrophy phenotypic changes in chondrocyte embedded in cartilage play a critical role in the degradation of cartilage matrix. However, in RA, fibroblast-like synoviocytes (FLS) are the predominant cell type and contribute to the destruction of cartilage[37]. This might explain the findings from the present study that Col10neo, a degradation biomarker of type X collagen, is significantly higher in OA than RA.

The limitations of this study were: 1) the tissue-specificity of the serological Col10neo level. Although type X collagen is exclusively expressed by hypertrophic chondrocytes in cartilage, it has been found in human lumbar intervertebral discs [38, 39]. We previously reported that C-Col10, an assay measuring the C-terminus of type X collagen, was significantly higher in spondyloarthritis (SpA) patients due to the syndesmophyte outgrowth similar to osteophyte formation in OA[40]. Thus, the origin of serological Col10neo is worthy of further investigation. 2) Small sample size study. A study with larger sample size is needed to see if the findings from this study can be generalized. 3). The unequal sample size in different groups, which might minimize the statistical power. We noted that it was an exploratory research and cohorts with larger samples might be useful for validating this biomarker in the future research.

In conclusion, our findings raise the possibility that Col10neo linked to hypertrophic chondrocyte could be used as a biochemical marker for knee OA with a diagnostic potential in OA and RA, and also help get a better understanding of the role of hypertrophic chondrocytes in the pathogenesis of OA.

Author Contributions

MK, ACBJ, and YHE are in charge of overall direction and planning. YHE carried out all the experiments and took the leading role in writing manuscript. TMJ participated in monoclonal antibodies development. KKP and LAN contributed to the preparation of the C4Pain cohort. JSA, SAB, and MAT contributed to the preparation of the NYU progression cohort. TCH contributed to the human cartilage sample preparation. All authors contributed to and approved the final manuscript.

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Conflict of interest

MK, and ACBJ were full-time employees and shake holders of Nordic Bioscience A/S. YHE and TMJ are the full-time employee at Nordic Bioscience A/S. The rest co-authors have no conflict of interest to report.

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Figure Legends

1. **Schematic illustration of the positions of neo-epitope, collagenase cleavage site and antibody-binding sites on type X collagen. (A**)The red line is the location of neo-epitope recognized by 2F4 in the triple helix domain. The purple line is the binding site of 11G8 at the C-terminal of NC1 domain. The yellow line is the proposed cleavage site by collagenase in other studies. The C1q-like C-terminal NC1 domain forms a stable homotrimer. (**B)**The specificity of 2F4 tested by three synthetic peptides: selection peptide, elongated peptide, and truncated peptide, and two truncated recombinant type X collagen proteins: Col10 with free 479Gly (AA 479-680) and Col10 (AA 450-680), 30aa longer and spanning the cleavage site A^{478}-^{479}G.

2. ***in vitro* cleavage of cartilage by enzymes.** (**A**) The cleaved human cartilage by MMPs, ADAMTSs, and Cathepsins was applied to Col10neo assay. It has been shown that Cathepsin K was the only one among tested proteases to release the neo-epitope. The data were shown as mean±SD. (**B**) Western blot of human cartilage cleaved by Cathepsin K. Equivalent blots was probed with antibodies to 2F4 (left panels) or in the presence of selection peptide (middle panel) or elongated peptide (right panel) or 11G8 (the fourth panel).

3. **Immunolocalization of ^{479}GIATKG in cartilage.** (**A-C**). Histology with Safranin O/Fast green. A. The normal-arranged chondrocytes, smooth surface and mild degradation of superficial zone proteoglycan in first biopsy and B. less smooth surface and moderate degradation of proteoglycan in the cartilage matrix in the second biopsy. C. chondrocyte cluster, fibrillated surface, cleft and severe reduction in safranin O/Fast green was found. (**D-F**) immunostaining with the ^{479}GIATKG antibody. G-I immunostaining with C-terminus 11G8. (**J-L**) with non-sense mouse IgG as negative control. Scale bar in A-C=100µm; Scale bar in D-L=20µm.

4. **Association between KL grade and Col10neo level in the plasma of subjects in the C4Pain study**. The data were shown as mean±95CI%. (**A**) There was a trend toward a higher level of Col10neo in subjects with greater KL score, but do not reach statistical significance. (**B**) The greatest percent of subjects with KL3-4 was in the highest tertile of Col10neo. The one-way ANOVA with post-hoc Tukey-Kramer test was used. Plasma Col10neo data were logarithmic transformed in all analyses. A P value<0.05 was considered statistically significant.

5. **Plasma Col10neo levels in the NYU study.** (**A**)The data were shown as mean±95CI%. Col10neo was statistically higher in OA than control (p=0.0002) or RA (p<0.0001), and no significant difference was seen between control and RA. The one-way ANOVA with post-hoc Tukey-Kramer test was used. Plasma Col10neo were logarithmic transformed in all analyses. P value<0.05 was considered statistically significant. (**B**) ROC curve analysis. The area under the ROC curve (AUC) was 0.875 (95% confidence interval 0.806-0.9427; P<0.0001), indicating there was a good separation of Col10neo of two groups

Table 1. Demographics of subjects in the C4Pain study

KL grade	No. of women	No. Of men	Total	VAS grade	Age, years	BMI, kg/m^2
0-1	23	27	50	37±30	61.8±8.5	26.4±3.1
2	79	66	145	42±29	64.6±7.3	28.2±3.8
3	17	19	36	56±21	64.3±7.1	29.3±5.6
4	12	10	22	54±24	67.8±7.7$^{\$}$	29.5±3.9

Except where indicated otherwise, values are the mean ± SD. Vas grade=maximal pain intensity for the last 24 hour, BMI = body mass index.

$, P<0.05 compared to KL0-1 group

Table 2. Demographics of subjects in the NYU study

	Non-OA Controls	OA	RA
No. of men	12	56	6
No. of women	8	86	28
Total No.	20	142	34
Age, years	56.7±8.7$^{\#}$	62.5±10.1	53.2±10.5$^{\$\$\$\$}$
BMI, kg/m^2	26.75±4.14	26.58±3.62	27.99±5.70
KL score	≤1	KL 0-4	Not Available
Treatments	No	21.8 percent of the 142 enrolled OA patients were taking NSAIDs.	All enrolled 34 RA patients were taking vitamin or mineral supplements or medication treating high blood pressure. Besides, 34 percent were taking biologics (such as Orenica, Humira, and Enbrel); 71 percent were on methotrexate and among methotrexate patients 48 percent were on biologics.

Except where indicated otherwise, values are the mean ± SD. BMI = body mass index.
#, p<0.05 compared to OA group
$$$$, p<0.0001 compared to OA group

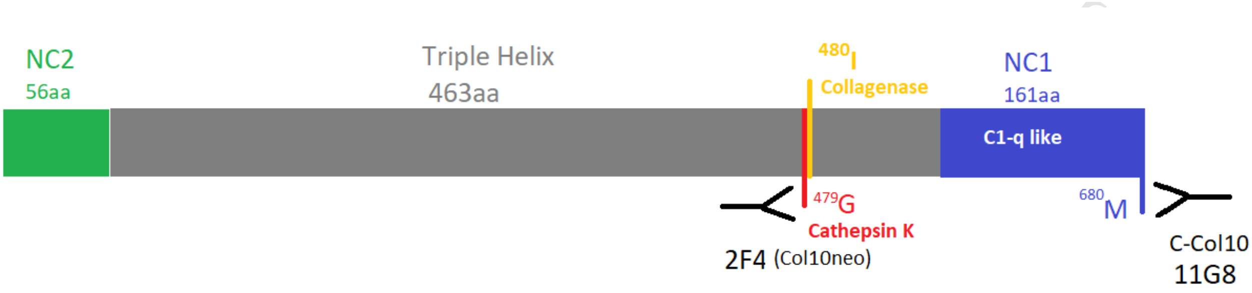
Table. 3 ANCOVA analysis of plasma Col10neo levels adjusted for age, gender, and BMI.

Factors			Mean difference	Std. Error	P [a]	95% CI a
OA	-	Non-OA control	0.1152	0.02926	0.0003	0.04453 to 0.1866
	-	RA	0.2176	0.02477	<0.0001	0.1578to 0.2774
Non-OA control	-	OA	-0.1152	0.02926	0.0003	-0.1859 to -0.04453
	-	RA	0.1024	0.03487	0.0112	0.01819to 0.04453

a Bonferroni corrected

Fig.1

A.


NC2
56aa
Triple Helix
463aa
480I
Collagenase
NC1
161aa
C1-q like
479G
Cathepsin K
680M
2F4 (Col10neo)
C-Col10
11G8

B.

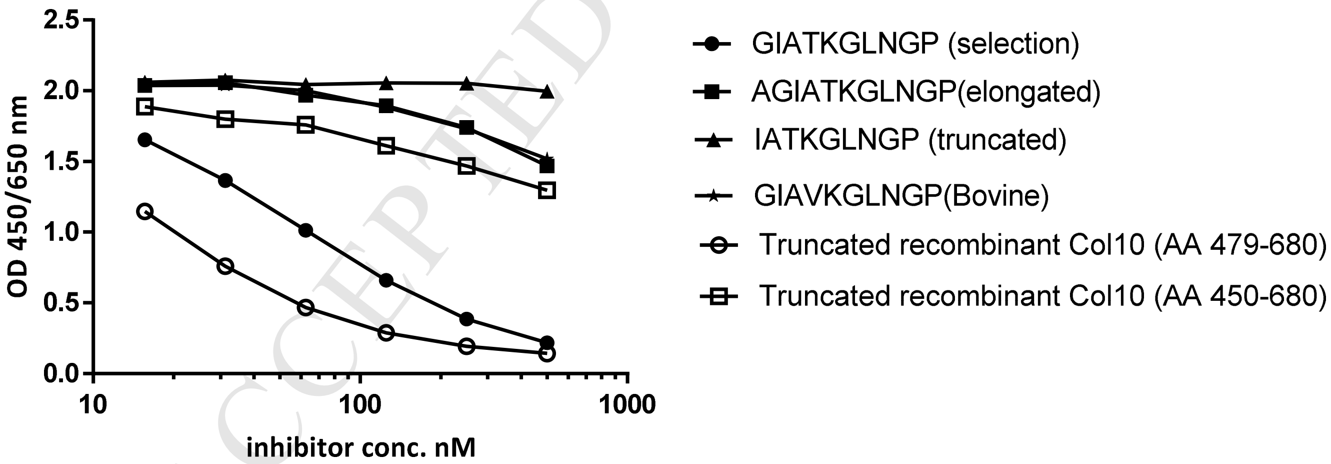

OD 450/650 nm
2.5
2.0
1.5
1.0
0.5
0.0
10
100
1000
inhibitor conc. nM
GIATKGLNGP (selection)
AGIATKGLNGP(elongated)
IATKGLNGP (truncated)
GIAVKGLNGP(Bovine)
Truncated recombinant Col10 (AA 479-680)
Truncated recombinant Col10 (AA 450-680)

Fig. 2

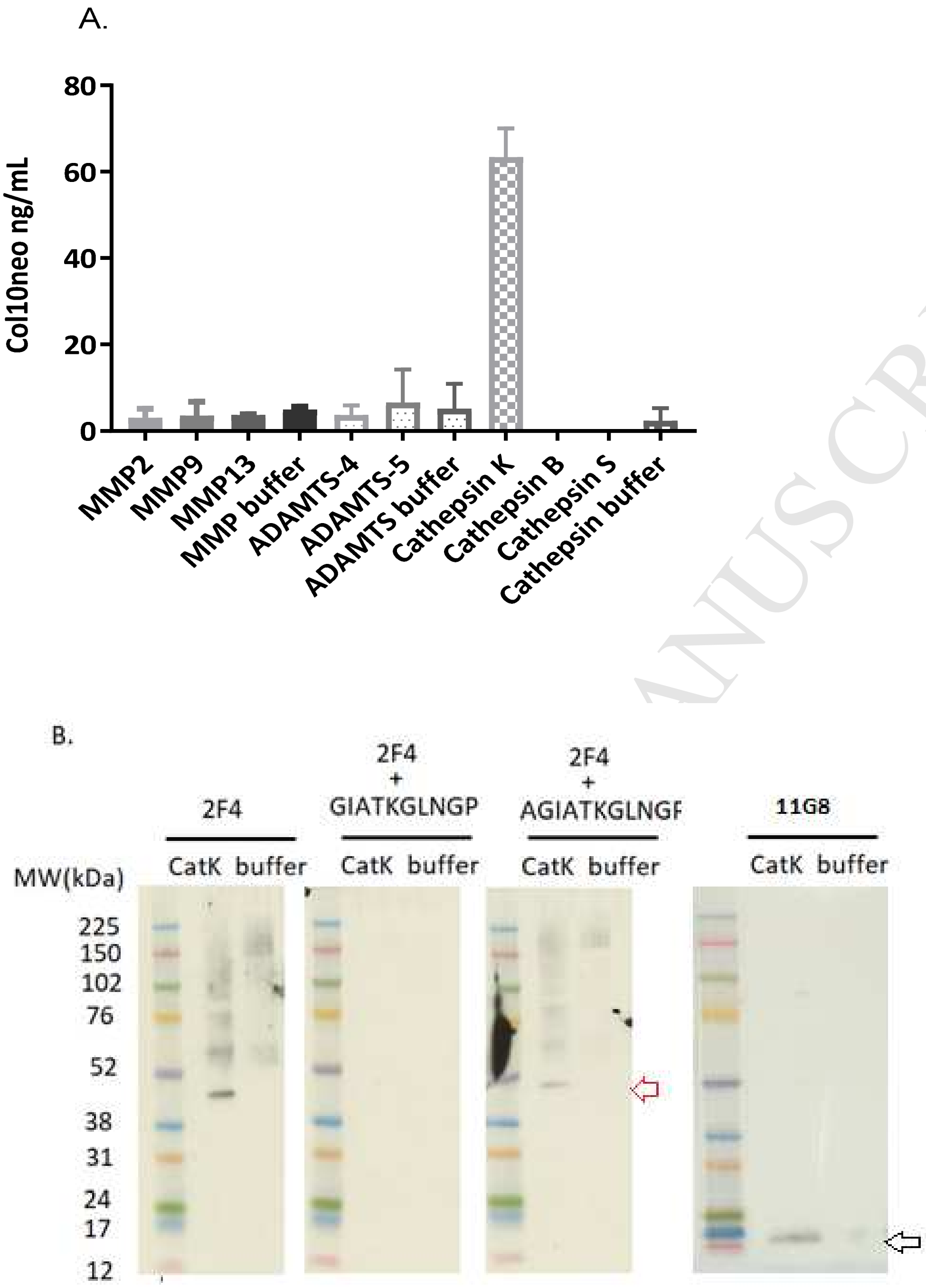

A.
Col10neo ng/mL
0
20
40
60
80
MMP2
MMP9
MMP13
MMP buffer
ADAMTS-4
ADAMTS-5
ADAMTS buffer
Cathepsin K
Cathepsin B
Cathepsin S
Cathepsin buffer
B.
2F4
2F4 + GIATKGLNGP
2F4 + AGIATKGLNGP
11G8
CatK buffer
CatK buffer
CatK buffer
CatK buffer
MW(kDa)
225
150
102
76
52
38
31
24
17
12

Fig3.

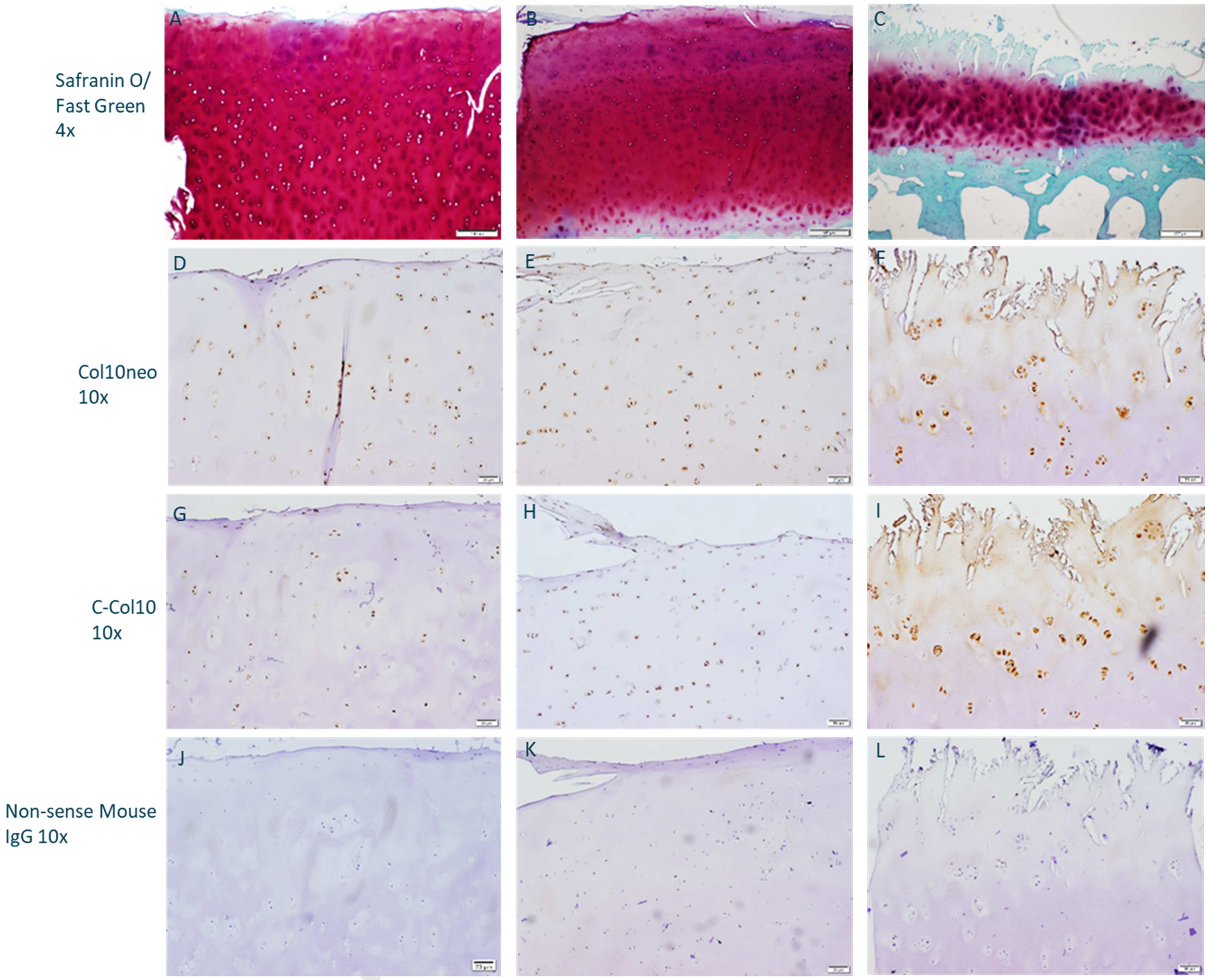
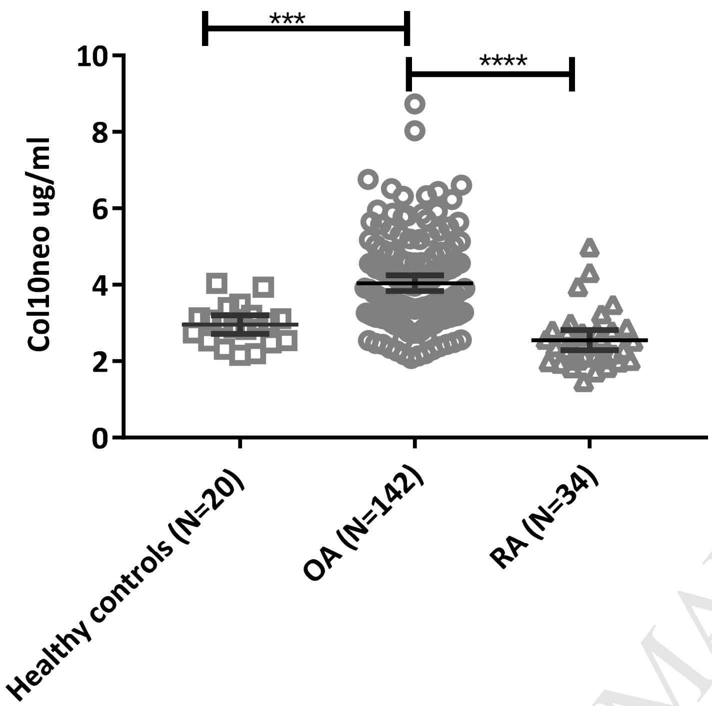

A
B
C
Safranin O/
Fast Green
4x
D
E
F
Col10neo
10x
G
H
I
C-Col10
10x
J
K
L
Non-sense Mouse
IgG 10x

Fig. 4

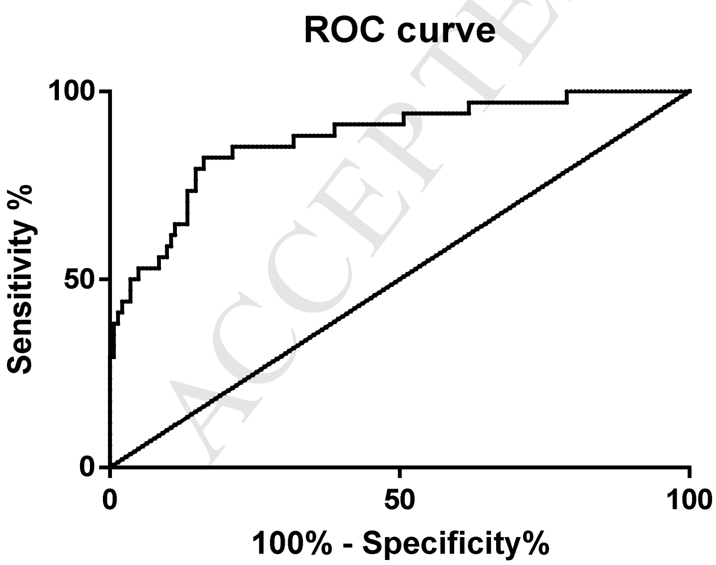

A.
Col10neo ug/ml
0
1
2
3
4
5
K/L0 (n=50)
K/L 2 (n=145)
K/L3 (n=36)
K/L4 (n=22)
B.
numbers of subjects
0
20
40
60
80
100
T1
T2
T3
Tertile of Col10neo levels
K/L3-4
K/L2
K/L0-1

Fig. 5

A.


***
****
10
8
6
4
2
0
Col10neo ug/ml
Healthy controls (N=20)
OA (N=142)
RA (N=34)

B.


ROC curve
100
50
0
Sensitivity %
0
50
100
100% - Specificity%