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Published in:
Osteoarthritis and Cartilage

DOI (link to publication from Publisher):
[10.1016/j.joca.2019.01.001](https://doi.org/10.1016/j.joca.2019.01.001)

Publication date:
2019

Document Version
Accepted author manuscript, peer reviewed version

[Link to publication from Aalborg University](#)

Citation for published version (APA):

He, Y., Manon-Jensen, T., Arendt-Nielsen, L., Petersen, K. K., Christiansen, T., Samuels, J., Abramson, S., Karsdal, M. A., Attur, M., & Bay-Jensen, A. C. (2019). Potential diagnostic value of a type X collagen neo-epitope biomarker for knee osteoarthritis. *Osteoarthritis and Cartilage*, 27(4), 611-620.
<https://doi.org/10.1016/j.joca.2019.01.001>

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Accepted Manuscript

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PII: S1063-4584(19)30017-2

DOI: <https://doi.org/10.1016/j.joca.2019.01.001>

Reference: YJOCA 4382

To appear in: *Osteoarthritis and Cartilage*

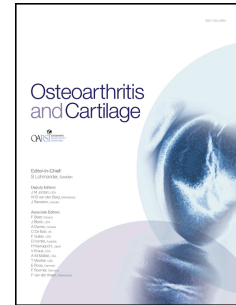
Received Date: 1 June 2018

Revised Date: 20 November 2018

Accepted Date: 7 January 2019

Please cite this article as: He Y, Manon-Jensen T, Arendt-Nielsen L, Petersen KK, Christiansen T, Samuels J, Abramson S, Karsdal MA, Attur M, Bay-Jensen AC, Potential diagnostic value of a type X collagen neo-epitope biomarker for knee osteoarthritis, *Osteoarthritis and Cartilage*, <https://doi.org/10.1016/j.joca.2019.01.001>.

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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

31 Abstract

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

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

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

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

50
51 **Key words:** chondrocyte hypertrophy, assay, biomarker, type X collagen, Osteoarthritis, Cartilage

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1 Introduction

2 Osteoarthritis (OA) is the most common joint disease, which is characterized by cartilage damage and
3 impaired joint function¹. It can implicate synovial joints, but knees, hands, hips, and spine are usually
4 affected by this disease². A range of risk factors, such as aging, gender, obesity, early trauma, and heredity
5 are associated with osteoarthritis of the knee³.

6 Although substantial progress has been made to understand the mechanisms leading to degradation of the
7 cartilage matrix in OA, the exact pathogenesis of OA still needs to be elucidated. Supported by emerging
8 evidence hypertrophy of chondrocytes, the only cells present in cartilage is a key event in the development
9 of OA^{4, 5,6,7,8}. The time course of expression of type X collagen, a specific marker of hypertrophic
10 chondrocytes, and MMP13 has shown to be significantly induced during OA progression in a surgically
11 induced joint instability OA model^{9,5}. In sections of human osteoarthritic cartilage, type X collagen was
12 found around hypertrophic chondrocyte clusters in the deep zone close to tidemark¹⁰. Also, Eerola et al.
13 demonstrated that intense immunostaining of type X collagen was observed specifically at sites of
14 osteophyte formation and surface fibrillation¹¹. Furthermore, the mineralization process has been shown in
15 OA cartilage indicating that articular chondrocytes from OA joints develop terminal differentiation similar
16 to those in the growth plate during endochondral ossification¹². Conclusively, chondrocyte hypertrophy is
17 associated with OA.

18 Cartilage degradation is a common feature for rheumatoid arthritis (RA), a systemic autoimmune disorder
19 as well. The degradation of cartilage matrix in both OA and RA is mediated by the overexpression of
20 enzymes, mostly of MMPs and ADAMTSs^{13,14}. However, the driving force behind the development of these
21 two disorders is different as summarized by Pap et al. in a review¹⁵. In OA, cartilage loss occurs as part of a
22 phenotypic shifting of chondrocytes, while in RA, cartilage damage is considered a result of activated
23 synovial fibroblast-like synoviocytes (FLS) and monocytes.

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

33 Methods

34 Materials

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

38 Selection of the sequence for immunization

39 Ten urine samples of peri/postmenopausal women with knee pain undergoing arthroscopy were analyzed
40 by GC/MS and the Mascot database was searched for type X collagen neo-epitopes. Seventeen peptide
41 sequences were found to be unique to human type X collagen. Two sequences carrying the same free c-
42 terminus located at amino acid (aa) position 478' (Accession No.: Q03692; UniProt) were discovered in the
43 urine from OA patients, indicating the cleavage occurring between the bond of A⁴⁷⁸-⁴⁷⁹G. The first 10 aa,
44 ⁴⁷⁹GIATKGLNGP⁴⁸⁸, of the free N-terminal end generated by this cleavage was selected for immunization.
45 Further, a sequence alignment with different species was conducted using the Basic Local Alignment Search
46 Tool (BLAST).

48 Monoclonal antibody (mAb) production

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

58 The mice with the highest antibody titer and best reactivity towards the specific peptide, GIATKGLNGP,
59 were chosen for fusion and boosted intraperitoneally (i.p.) with 100 µg of 100 µL KLH-conjugate three days
60 before fusion. The isolated splenocytes were fused with murine myeloma cells, SP2/0-Ag14 (ATCC®CRL-
61 1581™). Hybridoma cells were selected by using HAT (hypoxanthine-aminopterin-thymidine) medium.
62 Supernatants were screened against GIATKGLNGP-k (Biotin) Limiting dilution method was used to get
63 monoclonal antibody and isotype was tested with kit (5300-05, Southern Biotech). Hi Trap Protein G
64 column (17-0404-01, GE healthcare) was used for purification.

65

66 ⁴⁷⁹GIATKG specific immuno-assay (Col10neo)

67 A purified monoclonal antibody was first tested for specificity against three synthetic peptides 1) specific
68 peptide (GIATKGLNGP), 2) elongation of the specific peptide (AGIATKGLNGP), 3) truncation of the specific
69 peptide (IATKGLNGP), and two truncated recombinant type X collagen proteins [Col10(AA 479-680) and
70 Col10 (AA 450-680)] respectively in a competitive immunoassay. The optimized competitive immunoassay
71 protocol: a 96-well streptavidin pre-coated microplate was coated with 1ng/mlGIATKGLNGP-k(Biotin)
72 dissolved in 50mM PBS-BTB for 30 min at 20^oC. Then, the plate was washed five times with wash buffer (20
73 mM Tris, 50 mM NaCl, pH 7.2). 20μL of standards,controls or samples were added to appropriate wells,
74 followed by 100μL of 23ng/ml 2F4in 50mM PBS-BTB buffercontaining 5% Liquid II (Roche Diagnostics,
75 Germany), and incubated overnight (20±1 hr) at 4^oC. After five times wash, 100μL of secondary antibody
76 (115-035-003, Jackson ImmunoResearch) was added and incubated for 1hr at 20^oC. The plate was washed
77 five times again. Finally, 100μL 3,3',5,5'-tetramethylbenzidine (TMB) was added and incubated for 15 min
78 at 20^oC in the dark. The colorimetric reaction was stopped by adding 100μL stopping solution (1% H₂SO₄)
79 and measured at 450nm with 650nm as the reference.

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

85

86 *In vitro* cleavage of human cartilage and western blot

87 Articular cartilage biopsies from OA patients who underwent knee replacement surgery were obtained
88 from Gentofte Hospital (Gentofte, Denmark). The collection and retrieval of the biospecimens complied
89 with international ethical guidelines for handling human samples and patient information. All participants
90 signed informed consent, and the study was approved by Danish authority. To identify the enzymes
91 responsible for the cleavage of the A⁴⁷⁸⁻⁴⁷⁹G bond in Col10, a knee cartilage pool of 10 OA patients
92 undergoing knee replacement surgery was cleaved by numerous proteases including matrix
93 metalloproteases-2, -9, -13 Cathepsins K, B, S, and A disintegrin and metalloprotease domains with
94 thrombospondins motifs-4, -5. Briefly, pulverized cartilage sample (30mg) was incubated with 1μg each
95 enzyme in a 0.5ml Eppendorf with 250μL digestion buffer as previously reported¹⁶. The digestion was
96 carried out for 24h in replicates. The reaction was quenched by adding EDTA (5mM) or E64 (5mM).

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

102

103 Immunohistochemistry (IHC)

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

114

115 Study participants

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

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

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

130

131 Statistics

132 Data were analyzed using GraphPad Prism 6 or MedCalc 16.8. For normality check, a Shapiro-Wilk test for
133 all variables of interest was performed. Between-group comparison for age, BMI and VAS score, the one-
134 way ANOVA with *post-hoc* Tukey-Kramer test was used. Plasma Col10neo data were logarithmic
135 transformed in all analyses. The ROC curve analysis was performed. The area under the curve was used to
136 determine how well Col10neo can distinguish between OA and RA groups. A P value<0.05 was considered
137 statistically significant. One asterisk (*) if $p<0.05$; two (**) if $p<0.01$; three (***) if $p<0.001$ and four (****) if
138 $p<0.0001$.

139

140

141 Results

142 Sequence alignment of the selected peptide across species

143 Two fragments sharing the identical C-terminal end, ⁴⁶³PGSKGDPGSPGPPGPA⁴⁷⁸ and
144 ⁴⁶⁵SKGDPGSPGPPGPA⁴⁷⁸ were identified by mass spectrometry in the urine samples of OA patients (Fig. 1A),
145 indicating the presence of a cleavage site existing between A⁴⁷⁸⁻⁴⁷⁹G. A 10aa length peptide from the free N-
146 terminus generated by the cleavage, ⁴⁷⁹GIATKGLNGP, has been chosen for immunization. The blast shows
147 the sequence is unique to human type X collagen. Sequence similarity across species shows 100% identity
148 between human and mouse, while a mismatched aa contained in rat or bovine compared to human
149 sequence.

150

151 Technical performance of the Col10neo assay

152 A monoclonal antibody 2F4 (isotype: IgG2b, κ) targeting the neo-epitope, ⁴⁷⁹GIATKG, was produced from a
153 hybridoma and purified by HiTrap Protein G affinity column. To test the specificity, the reactivity of 2F4
154 toward a biotinylated synthetic peptide, GIATKGLNGP-k(Biotin), completely displaced by adding of 500nM
155 selective peptide or equivalent amount of truncated recombinant type X collagen with free ⁴⁷⁹Glycine (AA
156 479-680). In contrast, a slight or no displacement of binding between the immobilized specific peptide and
157 2F4 was observed either with elongation/truncation of the specific peptide or cleavage-spanning
158 recombinant type X collagen (AA 450-680) at the same concentration (Fig. 1B). This indicated that 2F4 was
159 specific to the neo-epitope instead of intact type X collagen.

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

163

164 Cathepsin K-derived ⁴⁷⁹GIATKG

165 To investigate the responsible enzyme for cleaving Col10 A⁴⁷⁸⁻⁴⁷⁹G bond, various proteases at similar
166 concentration and incubation time were incubated with human cartilage and evaluated their relative
167 efficiency to generate the neo-epitope of ⁴⁷⁹GIATKGLNGP. We did not identify release of Col10neo in all
168 tested MMPs or ADAMTSs compared to untreated control. However, among the proteases, Cathepsin K
169 yielded substantial amount of Col10neo release (Fig. 2A). In an immunoblot, major band detected by 2F4
170 mAb in Cathepsin K degraded cartilage was ~50kDa representing the trimeric form of the NC1 domain. In a
171 completion Western blot, GIATKGLNGP peptide blocked 2F4 mAb recognition of fragment carrying the neo-
172 epitope of ⁴⁷⁹GIATKGLNGP. Furthermore, mAb 11G8 detected a 18kDa protein band which is most
173 likely the monomeric NC1 domain of type X collagen (Fig. 2B).

174

175 Immunolocalization of ⁴⁷⁹GIATKG in cartilage

176 To further define the distribution of ⁴⁷⁹GIATKG, serial sections of articular cartilage from 3 TKR (total knee
177 replacement) patients were stained with mAb 2F4 (Col10neo) and 11G8 (anti-C terminus of type X collagen)
178 and non-sense mouse IgG of cartilage. A representative histological images of damaged and undamaged
179 cartilage biopsies 1) the normal-arranged chondrocytes, smooth surface and mild degradation of superficial
180 zone proteoglycan (Fig. 3A), 2) loss of smooth surface and moderate loss of proteoglycan (according to
181 Mankin grading system²¹) (Fig. 3B) and 3) chondrocyte clusters, fibrillated surface, cleft and severe
182 reduction in safranin O/Fast green (Fig. 3C) were used for general assessment of degree of loss of
183 proteoglycan, chondrocyte morphology, and Col10neo expression. In mild and moderate degradation of
184 cartilage, GIATKG neo-epitope was localized to the peri-cellular matrix of chondrocytes, while in severe OA
185 its presence was extended to the territorial matrix of chondrocyte clusters and more prominent in
186 superficial fibrillation (Fig. 3D-F). The similar staining pattern was observed with C-terminus 11G8 mAb (Fig.
187 3G-I).. We did not observe any staining with non-sense mouse IgG (Fig. 3J-L).

188

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

190 The demographic characteristics of these four groups based on radiographic KL score are summarized in
191 table 1. Because the mean age of the participants was >60 years, few with a KL 0 and those with a KL of 1

192 were classified into the same group. 57% of the subjects involved in this study were with a KL of 2 and
193 assigned to group 2. There was no significant difference in gender distribution within each group. The mean
194 age of KL 4 group was significantly higher than KL 0-1 group ($p < 0.005$). There was a clear, but not
195 significant trend of increased BMI with increased radiographic grades.. There was a relationship between
196 the VAS score and K/L grade, but did not reach statistical significance.

197 The mean \pm 95CI% concentrations of Col10neo for in each KL groups: KL 0-1 2.6 [2.316-2.884] $\mu\text{g}/\text{mL}$, KL2
198 3.288[2.885-3.691] $\mu\text{g}/\text{mL}$, KL3 3.435[2.729-4.141] $\mu\text{g}/\text{mL}$ and KL4 3.517[2.599-4.435] $\mu\text{g}/\text{mL}$, respectively.
199 Although increased Col10neo levels associated with a higher KL grade, but it was not statistically significant
200 (Fig. 4A). However, when subjects were divided into tertiles based on Col10neo levels, we found that the
201 number of subjects with a KL 3-4 was greatest in the highest tertile of the Col10neo group (Fig. 4B).

203 Plasma Col10neo levels in the NYU study

204 The result from the C4Pain study encouraged us to investigate the potential use of Col10neo as a diagnostic
205 biomarker in another independent cohort of control and OA subjects. We utilized NYU Progression study
206 which consists of non-OA healthy control, OA, and RA. In this cohort, percentage of females was higher in
207 OA and RA groups than in the healthy control group. The mean age was significantly different in control and
208 OA groups ($P < 0.05$). However, the subjects were significantly younger in RA than in the OA group (Table 2;
209 $p < 0.0001$). There was no significant difference in BMI across groups.

210 The mean \pm 95CI% concentrations of Col10neo in control, OA and RA groups were 2.95 [2.71-3.19] $\mu\text{g}/\text{mL}$,
211 4.04 [3.84-4.25] $\mu\text{g}/\text{mL}$ and 2.55 [2.29-2.81] $\mu\text{g}/\text{mL}$, respectively (Fig. 5A). Plasma Col10neo level was
212 significantly higher in OA than healthy control ($p = 0.0002$) and RA ($p < 0.0001$). Tests of Between-Subjects
213 Effects have shown that there was an overall statistically significant difference in Col10neo levels between
214 different groups when adjusted for covariates ($p < 0.001$). After adjusting for covariates -age, gender, and
215 BMI, Col10neo level remained significantly higher in the knee OA patients compared to other two groups
216 (Table 3).

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

220 Discussion

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

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

226 Type X collagen is a short and non-fibril forming collagen containing three identical chains. Type X collagen
227 is well-recognized as a specific marker for hypertrophic chondrocytes²². It is synthesized primarily by
228 hypertrophic chondrocytes in the growth plate of fetal cartilage and believed to regulate bone
229 mineralization during endochondral ossification²³. The evidence for the up-regulation of type X collagen in
230 osteoarthritic cartilage on mRNA level or protein level by immunohistochemistry has been found in
231 multiple studies^{10,24,22,25,26}. In the current study, we have observed that type X collagen was stained
232 pericellularly with antibodies to the neo-epitope or C-terminus of type X collagen and distributed
233 extracellularly around chondrocyte clusters in the fibrillated superficial layer of osteoarthritic cartilage. This
234 is consistent with previous studies regarding the deposition of type X collagen by chondrocytes in OA
235 cartilage^{10,22}. Our findings confirm the notion of chondrocyte phenotype modulation in OA that
236 chondrocytes in diseased cartilage undergo terminal differentiation to hypertrophy. Moreover, some
237 characteristics of OA such as remodeling of extracellular matrix by proteases, vascularization and focal
238 calcification of joint resemble the physiological differentiation process of chondrocyte during skeletal
239 development^{4,27,7,12,6}.

240 The *in vitro* cleavage of human cartilage suggests that the neo-epitope of type X collagen, ⁴⁷⁹GIATKGLNGP,
241 was efficiently generated by Cathepsin K among a series of tested proteases. Collagenase is known to be
242 capable of cleaving type X collagen at least two cleavage sites, G¹⁵¹⁻¹⁵²I and G⁴⁷⁹⁻⁴⁸⁰I, within its triple-helical
243 domain^{28,29}. One site is near the amino-terminus of the triple helix, and another site is close to the carboxyl
244 terminus of the triple helix. As a consequence, these two cleavages generate three products, a 32-kDa
245 fragment containing the major triple-helix domain, a 9-kDa fragment from the amine-end of type X collagen
246 and another 18-kDa fragment containing the entire globular domain at the carboxyl end. Interestingly, the
247 newly identified cleavage site from human OA urine in the current study was verified to be generated by
248 Cathepsin K. This cleavage site at A⁴⁷⁸⁻⁴⁷⁹G bond is located an amino acid apart from the proposed
249 collagenase G⁴⁷⁹⁻⁴⁸⁰I. Not surprisingly, both bonds are in the same region where the Gly-Ile-Ala triplet was
250 disrupted by two additional amino acids from the next triplet. Because of the interruption, the region is
251 inevitably susceptible to attacks by a protease. Furthermore, increased expression and activation of
252 Cathepsin K has been reported in a transgenic mouse model for osteoarthritis and human osteoarthritic
253 cartilage^{30,31}. It is still uncertain whether cleavage of collagenases occurs prior to cleavage of Cathepsin K.
254 Further investigation is required. To sum up, our data suggest that Cathepsin K is potentially involved in the
255 turnover of type X collagen which is expressed by hypertrophic chondrocytes in OA.

256 Most interestingly, a recently published study has shown that a group of fragments containing the entire
257 C1q domain and variable portions of the attached linker and collagenous region designated as CXM, was a
258 degradation product of type X collagen during endochondral ossification and an ideal biomarker for the
259 overall growth plate activity³². The serum-based assay for CXM used in this study was built upon a
260 monoclonal antibody X34 specifically targeting a multimeric form of the NC1 domain, the ~50kDa NC1
261 region¹⁰. In the present study, a similar major band of 50 kDa was detected in the Cathepsin K-generated
262 human cartilage products by 2F4, a mAb specific to the neo-epitope ⁴⁷⁹GIATKGLNGP. It most likely consists
263 of the compact coiled C1q-like domain which has been reported to resist further proteolysis in other
264 studies^{33,34}. In our previous study, 11G8, an antibody raised against the last 10 amino acids at the carboxyl
265 end of type X collagen only recognizes the monomeric NC1 domain, ~18kDa¹⁷. Given that the dominant
266 form of the NC1 domain in circulation is an intact trimer, it is not surprising that Col10neo levels are
267 generally higher than C-Col10 in the same samples of clinical study C4Pain.

268 To the best of knowledge, it is the first time that a single biomarker has shown the possibility of
269 distinguishing OA from RA. Although both arthritis diseases affect joint, the molecular mechanisms of
270 cartilage breakdown are quite different in OA and RA. The role of terminally differentiated hypertrophic
271 chondrocytes in OA is still controversial. One study has shown the loss of expression of SOX-9, a
272 transcription factor inhibiting the differentiation progression of chondrocytes during endochondral
273 ossification, but a significant down-regulation of type X collagen gene expression in chondrocytes from OA
274 patients compared to age-matched controls³⁵. However, there is increasing evidence to support changes in
275 the phenotypic state of chondrocytes in OA. One important study demonstrated that the chemokines
276 were retained by binding to heparan sulfate proteoglycans to maintain chondrocyte resting phenotype. The
277 release of chemokines during cartilage degradation in OA contributes to chondrocyte phenotype shift³⁶.
278 Further damage is caused by proteolytic enzymes secreted by hypertrophic chondrocytes which in return
279 will lead to more loss of stability of chondrocytes, amplifying a vicious circle. To explain the discrepancy
280 between studies, there might be different patient subgroups existing as shown by the scattered Col10neo
281 levels in the OA group in the current study. The question if chondrocyte hypertrophy differentiation is a
282 generalized phenomenon in OA needs to be further investigated. RA is a chronic systemic inflammatory
283 disease instead, which is characterized by synovial lining cell proliferation, inflammatory cell infiltration,
284 and destruction of cartilage and bone. Therefore, the underlying molecular mechanisms behind the
285 pathogenesis of OA and RA may be different. We speculate that in OA, the hypertrophy phenotypic
286 changes in chondrocyte embedded in cartilage play a critical role in the degradation of cartilage matrix.
287 However, in RA, fibroblast-like synoviocytes (FLS) are the predominant cell type and contribute to the

288 destruction of cartilage³⁷. This might explain the findings from the present study that Col10neo, a
289 degradation biomarker of type X collagen, is significantly higher in OA than RA.
290 The limitations of this study were: 1) the tissue-specificity of the serological Col10neo level. Although type X
291 collagen is exclusively expressed by hypertrophic chondrocytes in cartilage, it has been found in human
292 lumbar intervertebral discs^{38, 39}. We previously reported that C-Col10, an assay measuring the C-terminus
293 of type X collagen, was significantly higher in spondyloarthritis (SpA) patients due to the syndesmophyte
294 outgrowth similar to osteophyte formation in OA⁴⁰. Thus, the origin of serological Col10neo is worthy of
295 further investigation. 2) Small sample size study. A study with larger sample size is needed to see if the
296 findings from this study can be generalized. 3). The unequal sample size in different groups, which might
297 minimize the statistical power. We noted that it was an exploratory research and cohorts with larger
298 samples might be useful for validating this biomarker in the future research.
299 In conclusion, our findings raise the possibility that Col10neo linked to hypertrophic chondrocyte could be
300 used as a biochemical marker for knee OA with a diagnostic potential in OA and RA, and also help get a
301 better understanding of the role of hypertrophic chondrocytes in the pathogenesis of OA.

302

303 Author Contributions

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

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310

311 Acknowledgements

312 Thanks to Tülay Dastan who did the monoclonal antibody production in Denmark. We also thank Helene
313 Sofie Hector for picking up human cartilage samples at the hospital. The study was funded by the Danish
314 Research NIH funded study (R01-AR- -052873) to SBA.

315

316

317 **Conflict of interest**

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

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- 431

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 A⁴⁷⁸-⁴⁷⁹G.
- 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 ⁴⁷⁹GIATKG 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 ⁴⁷⁹GIATKG 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 Orencia, 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

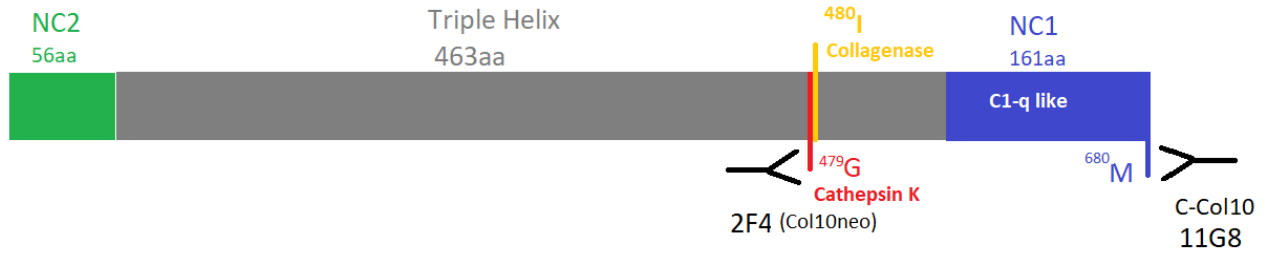
^{\$\$\$\$}, p<0.0001 compared to OA group

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.



B.

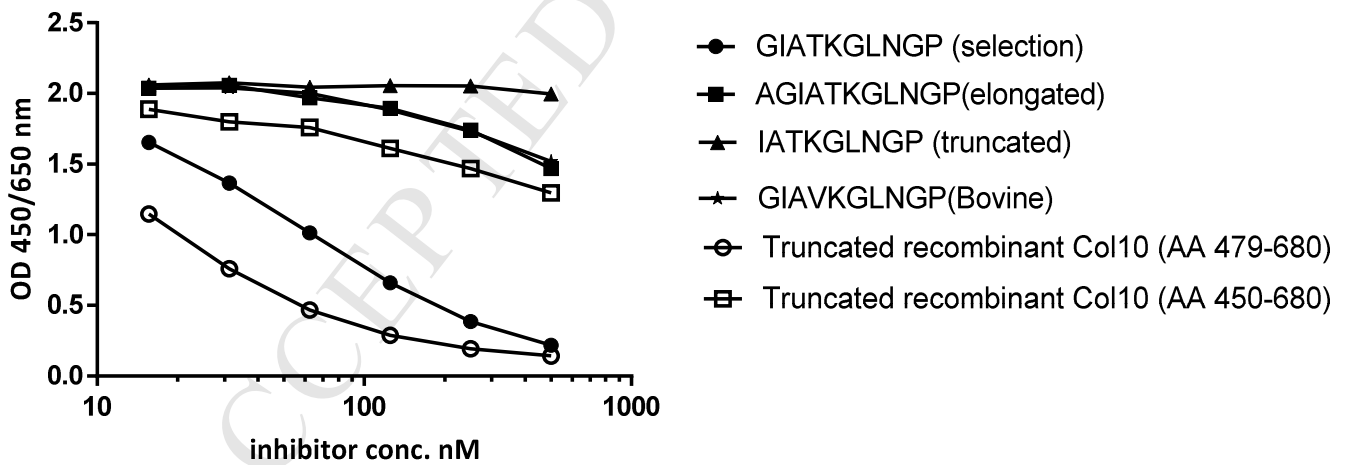


Fig. 2

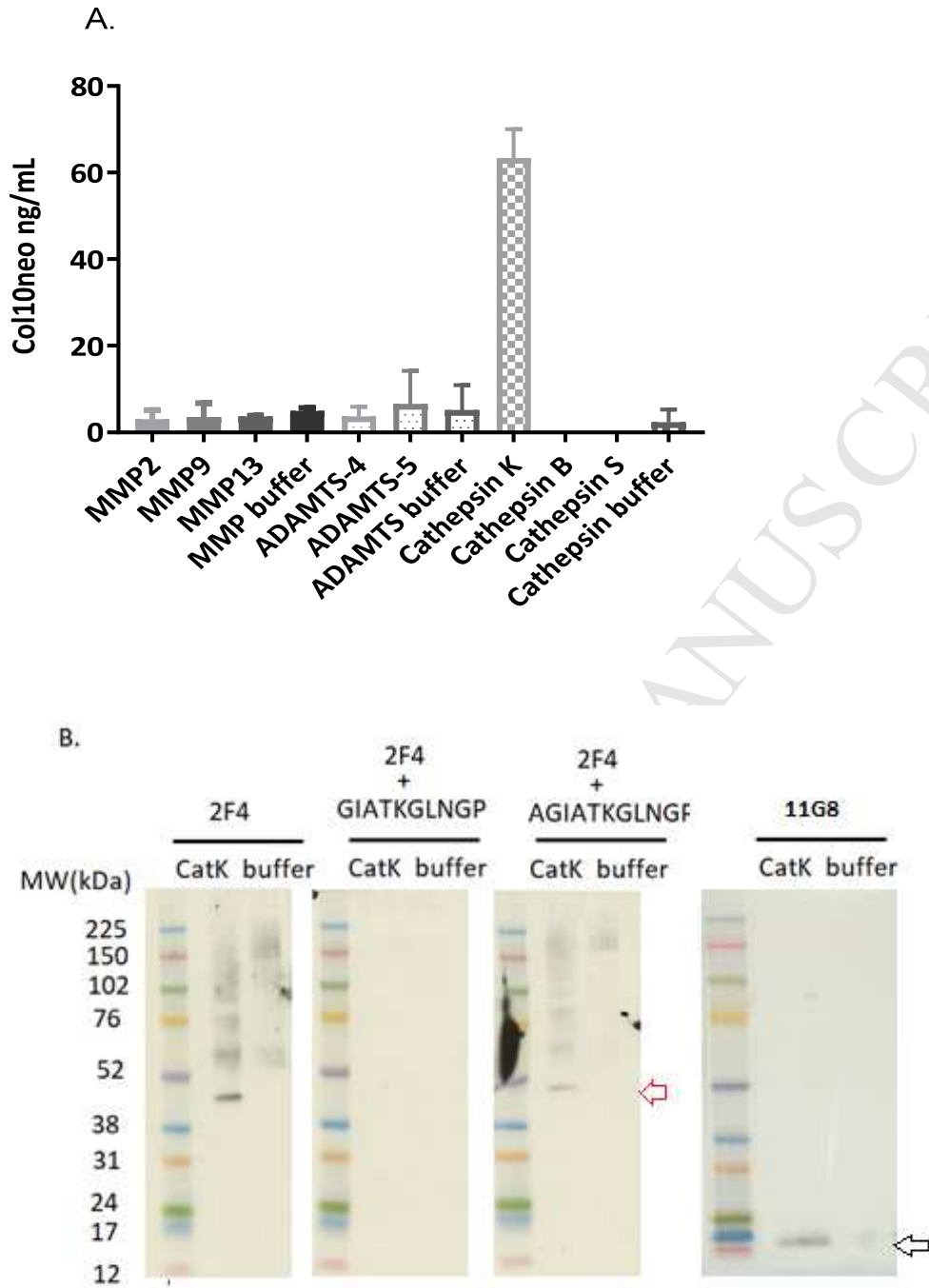
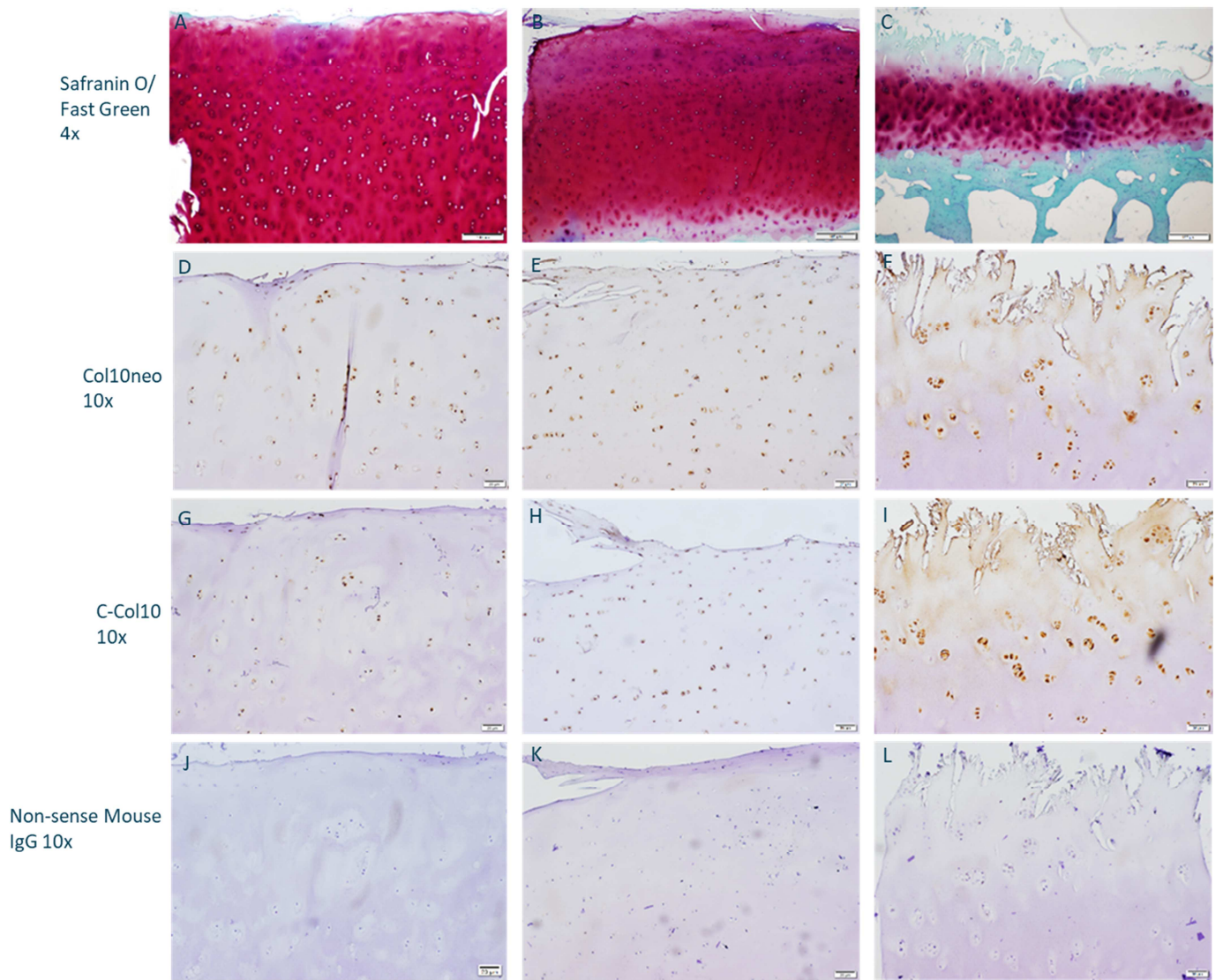


Fig3.



ACCEPTED

Fig. 4

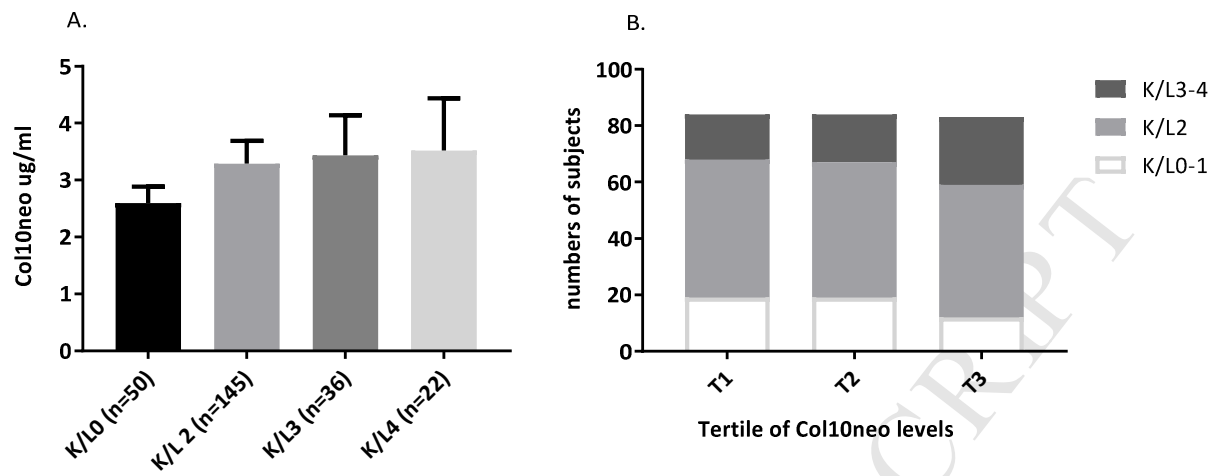


Fig. 5

