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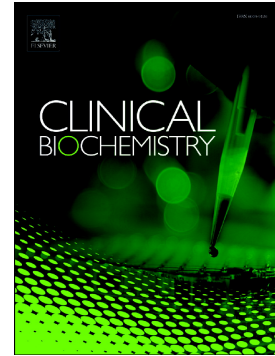
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Aggrecanase degradation of type III collagen is associated with clinical knee pain

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ABSTRACT

There is a lack of biochemical markers for non-invasive and objective assessment of symptomatic osteoarthritis (OA). Aggrecanase activity has been shown to be associated with joint deterioration and symptomatic disease through the degradation of extracellular matrix proteins, such as type III collagen. Our study aimed to identify and develop a novel biomarker by measuring an aggrecanase-mediated type III collagen neoepitope, and correlate levels of this biomarker with OA joint pain. Mass spectrometric analysis of purified type III collagen, degraded by the aggrecanase *A Disintigrin and Metalloproteinase with Thrombospondin motif* (ADAMTS), revealed a fragment generated by ADAMTS-1, -4 and -8. A monoclonal antibody was raised against the neoepitope of this fragment (COL3-ADAMTS) and a competitive ELISA was developed and tested; using serum samples from a cross-sectional cohort of patients with different degrees of knee OA (n=261). The COL3/ADAMTS ELISA was technically robust and specific for the ADAMTS-1, -4 and -8 generated neoepitope. COL3/ADAMTS was released form cytokine stimulated synovial cultures, indicating a biologic link between the marker and synovium. In OA patients, serum COL3/ADAMTS was independently associated with pain scores (rho=-0.13-0.17, p<0.05). This association was associated significantly with the presence of radiographic OA. Together, these data indicate that COL3/ADAMTS could be a marker of early osteoarthritis and the underlining pathology.

Keywords: Biochemical markers, type III collagen, pain, OA, ADAMTS, protease-cleaved neoepitope.

Keywords: Type III collagen, ADAMTS, Knee osteoarthritis, neoepitope, biochemical marker

ABBRIVIATIONS

ADAMTS   *A Disintigrin and Metalloproteinase with Thrombospondin motif*

COL3   type III collagen

ECM   extracellular matrix

OA   osteoarthritis

VAS   visual analog score

HIGHLIGHTS

- That a technical robust ELISA, COL3/ADAMTS, was developed which show good sensitivity for measurement in human serum samples
- COL3/ADAMTS was released from human osteoarthritis synovial membranes cultured is the presence of proinflammatory cytokines, indicating a link to synovial pathology and inflammation
- When the marker was measured in serum of 261 subjects with or without knee pain, we found that it was significantly and inversely associated with radiographic knee osteoarthritis.

1   INTRODUCTION

Osteoarthritis (OA) is characterized by a gradual deterioration of articular joint cartilage, along with remodeling of subchondral bone and synovial inflammation. The process is partly driven by an upregulation of aggrecanases and matrix metalloproteinases (MMPs), which degrade and remodel the extracellular matrix (ECM) of joint tissues [1]. The ECM mainly consists of collagens, proteoglycans and glycoproteins, all of which play important and unique functions in maintaining the physicochemical structure of tissue [1-3]. ECM remodelling is a normal process in which formation and degradation of healthy tissue is balanced, however an imbalance exists in disease, which manifests itself in various pathologies. Degradation of the cartilage by the aggrecanase, A Disintigrin and Metalloproteinase with Thrombospondin motif (ADAMTS), is well described [2][3]; whereas the role of ADAMTS in the turnover of the fibrous synovial tissue, is less so. Increased ADAMTS activity has been found in the synovial fluid of OA patients[4], thus the action of these enzymes may not only be restricted to the cartilage.

The fibril-forming type III collagen is mainly associated with type I collagen, and together they provide the structural framework of most connective tissues, including the synovial membrane which surrounds synovial joints [5]. Type III collagen contributes to synovial tissue elasticity, a property that is uniquely connected to this type of collagen [6]. Type III collagen is also found in healthy cartilage and is thought to stabilize the type II collagen framework [7].

The pathogenesis of OA involves an inflammatory response, and the activation of chondrocytes, fibroblasts and leukocytes partly mediates tissue turnover by the secretion of MMPs and ADAMTSs [3]. Type III collagen is upregulated in response to pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β)[8] which results in thickening of the synovial membrane. In addition, type III collagen has been found to be elevated in osteoarthritic cartilage [9]. Pro-inflammatory cytokines also upregulate the release of ADAMTS from articular cartilage leading to degradation of both cartilage and synovial membrane ECM [10,11]. Collagen fragments are released into circulation and can potentially be assessed as markers of joint degradation in synovial fluid, serum and urine. Such protein fragments, referred to as neoepitopes or protein fingerprints [12,13], have proven to be more accurate than their intact protein of origin, in detecting and quantifying certain pathophysiological processes [14]. For example, fragments of type II collagen, generated by MMPs, have been shown to be markers of cartilage degradation and joint deterioration in OA and rheumatoid arthritis (RA) [15].

The aim of the present study was to develop a novel ELISA specific for an ADAMTS generated fragment of type III collagen. In addition, we determined whether ADAMTS mediated degradation of type III collagen may be useful for monitoring joint type III collagen turnover in OA, along with its relationship with symptomatic disease, particularly pain and function.

2   MATERIALS AND METHODS

2.1   Patient samples

Serum COL3/ADAMTS levels were measured in a total of 261 subjects belonging to the cross-sectional C4P-003 study [16]. Subjects had different degrees of primary knee OA defined by different degrees of knee pain (maximal pain during the last 24 hours rated on a visual analog scale [VAS]) (VAS 0-100), and different degrees of radiological knee OA (Kellgren and Lawrence grade (KLG)). Radiographic knee OA (RKOA) was defined as having a KLG of 2 [17]. Only subjects which had a complete set of matching serum, X-rays and questionnaires available were included in the current investigation (261 out of 282 subjects with available serum samples)[18]. Subjects were segregated into four groups based on their VAS pain score: no pain: VAS <10 (n = 52), mild pain: VAS 10 to 39 (n = 81), moderate pain: VAS 40 to 69 (n = 67) and severe pain: VAS 70 to 100 (n = 61). Age, BMI, and disease duration (if any), as well as WOMAC [19] and Lequesne [20] scores were collected (table 1). All subjects were Caucasian and abstained from analgesic medication for at least 72 hours [16]. The study was approved by the Danish ethical committee (VEK; The Ethical committee of North Jutland no.: N-20100094).

2.2   Reagents

All reagents used for experiments were standard high-quality chemicals from companies such as Merck (Whitehouse Station, NJ, USA) and Sigma Aldrich (St. Louis, MO, USA). The synthetic peptides used for monoclonal antibody (mAb) production and assay development were purchased from the Chinese Peptide Company (Beijing, China).

2.3   In vitro cleavage

Purified type III collagen from human placenta (Abcam, Cambridge, UK) was cleaved with pro-ADAMTS-1,-4, and -8 and MMP-9 (Calbiochem, Merck, Whitehouse Station, NJ, USA). 50 µg pro-ADAMTS' or MMP-9 was activated with 20 µL 1 mM 4-aminophenylmercuric acetate (APMA) in dimethyl sulfoxide

(DMSO) and incubated at 37°C for 2 hours. 1 mg/mL type III collagen, diluted in 0.5 M acetic acid, was dialyzed into MMP buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl$_2$, 2 mM Zn acetate, pH 8.0) for two days at 4°C, and proteins below 10 kDa removed by filter centrifugation (Microcon Ultracel YM-10, cat. no. 42407, Millipore, Billerica, MA, USA). Each digest was performed separately by mixing 100 µg type III collagen with 1 µg of enzyme (ADAM-TS -1, -4, -8 or MMP-9) in MMP buffer (n=4). As a control, 100 µg of type III collagen was mixed with MMP buffer only. All digests were incubated for 24 h at 37°C and terminated using 5 mM EDTA. Cleavage with cathapsin K and S was done in 25 mM NA$_2$HPO$_4$, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, pH 6.5 at 37°C and the digest was stopped after 24 h 1 µM cysteine protease inhibitor E64 (cat.no.219377, Calbiochem, Merck, Whitehouse Station, NJ, USA). Collagen cleavage was verified by SDS-PAGE separation, and visualized using SilverXpress® Silver Staining Kit (cat. no. LC6100, Invitrogen, Carlsbad, Ca, USA) according to the manufacturer's instructions.

2.4 Peptide identification

Peptide fragments of in vitro cleaved type III collagen were identified using liquid chromatography coupled to electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS). Type III collagen/ ADAMTS digests were ultra-filtrated to remove proteins above 10kDa, the pH adjusted to 2.0 using formic acid and 4 µL of sample was analyzed by LC-MS/MS. LC was performed on a nanoACQUITY UPLC BEH C18 column (Waters, Milford, MA, USA) using a formic acid/acetonitrile gradient. MS and MS/MS were performed on a Synapt High Definition Mass Spectrometry quadruple time of flight MS (QUAD-TOF; Waters, Milford, MA, USA), with an acquisition range of 350-1600 m/z in MS and 50-2000 m/z, in MS/MS. The software "ProteinLynx Global SERVER (PLGS)" (Waters, Milford, MA, USA) was used to analyze spectra and generate peak lists. To identify peptides, MS and MS/MS data was searched against a type III collagen (FASTA) protein database using the Mascot 2.2 (Matrix Science, Boston, MA, USA) software with ESI-QUAD-TOF settings with carbamidomethyl (C), oxidation of methionine (M), oxidation of lysine (K) and oxidation of proline (P) as variable modifications. Only sequences identified with a significant Mascot score were regarded further.

2.5 Selection of peptide for immunizations

The first six amino acids of collagen fragment free ends, identified by MS, were regarded as a neoepitope generated by each ADAMTS in question. All protease-generated sequences were analyzed for homology and distance to other cleavage sites, then blasted for homology using the NPS@: network

protein sequence analysis [42]. Six type III collagen sequences, identified in fragments generated by ADAMTS cleavage, were selected for immunizations.

2.6 Immunization procedure

Six 4-6 week old Balb/C mice were immunized subcutaneously in the abdomen with 200 µL emulsified antigen (50 µg per immunization) per immunization program, using Freund's incomplete adjuvant. Immunizations using the following immunogen provided a neoepitope specific antibody: GAPGFRGPAG-CGG-KLH (American Peptide, Hongkong, China). Immunizations were performed at two-week intervals until stable titer levels were obtained. At each bleeding, the serum titer was investigated and the mouse with the highest titer was selected for fusion. The selected mice were boosted intravenously with 50 µg immunogen in 100 µL 0.9% sodium chloride solution three days before isolation of the spleen for cell fusion. Only one immunogen resulted in satisfactory immune response; the remaining programs were terminated.

2.7 Fusion and antibody screening

The fusion procedure has been described elsewhere [43]. Briefly, mouse spleen cells were fused with SP2/0 myeloma fusion partner cells. The hybridoma cells were cloned using a semi-solid medium method and transferred into 96-well microtiter plates for further growth. Here, standard limited dilution was used to promote monoclonal growth. Supernatants were screened using an indirect ELISA, while the biotinylated peptide GAPGFRGPAG-K-Biotin (American Peptide, Hongkong, China) was used as a catcher peptide on streptavidin-coated microtitre plates.

2.8 Characterization of clones

Native reactivity and peptide binding of mAbs was evaluated in a preliminary competitive ELISA. mAbs in hybridoma supernatant competed with samples of human, rat or mouse serum and plasma, for biotinylated peptide (5 ng/mL), bound to a streptavidin coated microtitre plate. Clones were also tested against the free synthetic peptide (GAPGFRGPAG), a non-sense synthetic peptide, and a synthetic peptide elongated with one amino acid at the free N-terminal end (RGAPGFRGPAG). Isotyping of the mAbs was performed using the Clonotyping System-HRP kit, cat.5300-05 (Southern Biotech, Birmingham, AL, USA). The selected clones were purified using Protein G columns according to manufacturer's instructions (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK).

2.9   COL3/ADAMTS ELISA protocol

We labeled selected mAbs (5D9) with horseradish peroxidase (HRP) using the Lightning link HRP labeling kit according to the instructions of the manufacturer (Innovabioscience, Babraham, Cambridge, UK). A 96-well streptavidin plate was coated with biotinylated synthetic peptide GAPGFRGPAG-K-Biotin dissolved in assay buffer (25 mM PBS, 1% BSA, 0.1% Tween-20, pH 7.4 adjusted at 4^oC) and incubated 30 minutes at 20°C. 20 µL of free 10 amino acid peptide calibrator (GAPGFRGPAG) or sample were added to appropriate wells, followed by 100 µL of HRP conjugated mAb and incubated 1 hour at 20°C. Finally, 100 µL tetramethylbenzinidine (TMB) (Kem-En-Tec cat.438OH) was added and the plate was incubated for 15 minutes at 20^oC in the dark. All the above incubation steps included shaking at 300 rpm. After each incubation step, the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The TMB reaction was stopped by adding 100 µL of stopping solution (1% HCl) and measured at 450 nm with 650 nm as the reference. A calibration curve was plotted using a 4-parametric mathematical fit model.

2.10 Technical evaluation

From 2-fold dilutions of quality control (QC) samples consisting of serum and plasma samples, linearity was calculated as a percentage of recovery of the 100% sample. The lower limit of detection (LDL) was determined from 21 zero samples (i.e. buffer) and calculated as the mean plus three standard deviations. The inter- and intra-assay variation was determined by 10 independent runs of six QC samples. The developed COL3/ADAMTS ELISA was evaluated using protease digests described under "In vitro cleavage". 20 µL from a sample diluted 1:20 was used for the assay.

2.11 Explant cultures

Human synovial tissue and cartilage used for ex vivo cultures was collected from OA patients undergoing total knee replacement at Gentofte Hospital, Denmark. All patients gave written informed consent (Danish science ethical committee, amendment no. 53713, approval no. H-D-2007-0084). Synovial tissue and cartilage were stored separately until receipt. Human synovial membrane samples were cleaned of visible fat, and cut into explants (hSMEx) of 30 ± 5 mg. Human cartilage was punched into 5 mm explants (HEX) with a biopsy punch (Miltex, MTP-33-35). hSMEx were co-cultured with HEX at a one-to-one ratio, or cultured separately as mono-cultures. All explants were cultured in Dulbecco's Modified Eagle

Medium supplemented with F12, GlutaMAX™ and 1% penicillin and streptomycin (Sigma-Aldrich, Denmark) in 48 well plates for 14 days; without treatment (W/O) or with TNFα [20ng/mL] and oncostatin M (OSM) [10 ng/mL] (O+T). Conditioned media was removed and fresh treatment added every 2-3 days. Undiluted conditioned media was used for the measurement of COL3/ADAMTS. Synovial tissue from two patients and cartilage from four patients was used. Synovial tissue and cartilage from the same patient were co-cultured. However due to insufficient amount of cartilage, the synovial tissue was also co-cultured with cartilage from another patient. A total of six replica were included in the experiment.

2.12 Statistical analysis

Results are shown as mean with 95% confidence intervals, SEM or SD. Differences between mean values were compared by either Mann-Whitney's t-test for two-tailed observations or ANCOVA adjusting for covariates (age, gender, BMI and smoker status (yes/no)). The P values were Bonferroni corrected. Associations between the marker COL3/ADAMTS and clinical pain scores were tested weight least squares multiple regression (AutoWeight $1/SD^2$) adjusting for the covariates. Odds ratio (OR) and area under the curve (AUC) were calculated by logistic regression including the covariates. P values less than 0.05 were considered significant. Statistical analyses were performed Medcalc® (version 17.2) and plots were done by using GraphPad Prism (version 7).

3   RESULTS

3.1   Identification of ADAMTS generated type III collagen fragments

A large number of type III collagen fragments with statistically significant Mascot score (p<0.05) were identified after in vitro cleaved by ADAMTS -1, 4, or -8. The protease-generated neoepitopes were tested for homology between human and rat. The sequence 486'⇓GAPGFRGPAG'495 (C3A) in the alpha 1 chain of type III collagen, generated by ADAMTS -1, -4 and -8 (⇓), was selected for immunizations since it is 100% homologous to human and rat type III collagen.

3.2 COL3/ADAMTS ELISA development, characterization and technical performance

An IgG1 mAb was produced, selected and used for the development of a solid-phase competitive ELISA, COL3/ADAMTS. The ELISA signal was completely inhibited by 250 ng/mL of specific neoepitope peptide, whereas an elongated peptide inhibited the signal by less than <10% at the same concentration (Fig. 1A). A nonsense peptide did not inhibit the signal (Fig. 1A). Type III collagen cleaved by different ECM associated proteases was tested in the COL3/ADAMTS ELISA to verify the sensitivity towards the target epitope. It was observed that ADAMTS -1, -4, and –8 were able to generate the COL3/ADAMTS fragment (Fig. 1B), with ADAMTS-4 generating by far the most. There was little or no reactivity towards intact human type III collagen or fragments generated by the cathepsins and MMPs tested. These findings were consistent in two repeated cleaved batches.

The assay specifications are summarized in Table 2. Briefly, the assay range was between 2 and 60 ng/ml and linearity was within 100 ± 15% for human serum down to an 8-fold dilution (table 2). The inter- and intra-assay variations, using human serum and urine QC samples, were both below 10% (table 2).

3.3   Biological target validation

COL3/ADAMTS was measured in the conditioned media of human osteoarthritic cartilage explants (HEXs), synovial membrane explants (hSMEx), and co-cultured explants, over the course of 14 days using the COL3/ADAMTS ELISA. COL3/ADAMTS was released from co-cultures, and hSMExs alone, with O+T stimulation in a time-dependent fashion, with peak release at day 3 (Fig. 2A). Patient associated variance was observed, especially with hSMExs, however release of COL3/ADAMTS was 3-fold higher from co-cultured explants after 14 days of 0+T stimulation, compared to unstimulated controls (w/o), (p<0.05, Fig. 2B) .

3.4 Association between serum COL3/ADAMTS and patient reported pain in OA

The average concentration of COL3/ADAMTS in serum collected from 261 subjects with or without knee pain was 21.8 [19.4 to 24.0] ng/ml. Serum COL3/ADAMTS levels were weakly, but significantly, inversely associated with pain; determined by VAS (r=-0.15, p<0.05), WOMAC (r=-0.17, p<0.01) and Lequesne (r=-0.13, p<0.05) assessment, following adjustment for age, BMI and gender (Table 3). There was no association between COL3/ADAMTS levels and WOMAC function or stiffness. There was no linear correlation between KL grade and COL3/ADAMTS (p_{ANOVA}>0.1, data not shown).

Subjects were separated into four sub-categories based on their VAS pain level, followed by separation into those with or without radiographic knee OA (RKOA) (Table 4). There was a significant difference in serum levels of COL3/ADAMTS between subjects with no pain, and subjects with mild (p=0.0076) or moderate pain (p=0.0019), when RKOA was not considered. Within the RKOA group, COL3/ADAMTS was significantly elevated in patients with mild pain (p=0.0086) but there was no difference between non-RKOA and RKOA patients; thus COL3/ADAMTS levels were not associated with radiographic knee OA, but rather with pain status.

Next, the performance of COL3/ADAMTS was compared to other markers of ECM degradation and joint pathology, which have previously been published [18][21]. COL3/ADAMTS could significantly predict who would had symptomatic knee OA versus those without (Table 5), with an odds ratio of 0.56 demonstrating a significant inverse relationship (p= 0.0003). Another marker of type III collagen degradation, C3M, which is derived from increased metalloproteinase activity, also inversely correlated with symptomatic RKOA and performed better than COL3-ADAMTS; AUC of 0.78 vs 0.72 (Table 5). An ADAMTS derived fragment, AGNx1, a metabolite of aggrecan, positively correlated with symptomatic RKOA but performed worse than COL3-ADAMTS; 0.65 vs. 0.72. COL3/ADAMTS performed better than age and BMI alone (Table 5).

4   DISCUSSION

In this work, we present the identification and development of a novel ELISA-based marker, which measures human type III collagen cleavage derived from the activity of ADAMTS. The work showed that a sensitive monoclonal antibody was produced, which specifically recognized the collagen type III neoepitope, COL3/ADAMTS, and not an elongated or nonsense peptide. The assay was technically robust and sufficiently sensitive to detect COL3/ADAMTS in patient serum. We also found that the neoepitope was released from human OA synovial membrane when culture in the presence of proinflammatory cytokines, indicating that the marker was indeed associated with joint tissue turnover and could be relevant as a marker for joint health.

4.1   The association between COL3/ADAMTS and OA pain

We tested the ELISA in a cross-sectional cohort, which included 261 subjects with varying degree of symptomatic and radiographic OA. We found that serum levels of COL3/ADAMTS significantly and inversely correlated with patient pain score (r≈ -0.15). Moreover, that this association was found to be related to symptomatic radiographic OA rather than knee pain alone (non-radiographic OA). The correlation was weak (r<0.2) which indicates that COL3/ADAMTS is not a strong surrogate marker of pain severity. However, it may provide insight to the underlining pathogenesis of ECM remodeling in OA and its association with development of pain, which is a key diagnostic feature in OA [22]. Lastly, we compared COL3/ADAMTS with other synovial or cartilage markers of either type III collagen (C3M [23]) or aggrecanase activity (AGNx1 [24]). We found that low level of COL3/ADAMTS was predictive of symptomatic radiographic OA with an odds ratio of 1.8 (1/0.56, p=0.0003, table 5). This was slightly better than high level of AGNx1 which had an odds ratio of 1.8, but borderline significant (p=0.066), but not as significant as low C3M, which predicted with an odds ratio of 2.5 (1/0.40, p<0.0001).

Pain in OA is considered multimodal and this weak correlation may indicate that type III collagen degradation reflects only part of the underlining mechanism observed in knee pain. Interestingly, the correlation was independent of radiographic disease severity, which further supports the idea that radiographic OA and pain in OA are not strictly associated [22]. Our results point to the possibility that changes in the turnover, both elevated and decreased, of joint connective tissue is associated with increased structural severity of OA, which cannot be described by pain or demographics alone. We

speculate that COL3/ADAMTS is a potential biomarker for the identification of patients with minimal symptomatic knee OA, but with early and potentially progressing OA.

The pain intensity reported by OA patients seems to be only weakly correlated with radiological score, suggesting factors other than cartilage destruction and bony changes are important in OA pain [25]. Recent studies have investigated specific groups of patients with OA, characterized by high pain intensities but low radiological severity, and these patients seems to be highly sensitive to pain [16,26]. Further, low radiological severity before total knee replacement (TKR) has been linked to low functionality after surgery and recently, low radiological scores in combination with pain sensitivity profiles were found associated with less pain relief following TKR [27]. The current study found COL3/ADAMTS to be associated with pain but not radiological scores, which further supports that radiological OA, might be less important compared with clinical pain.

4.2 COL3/ADAMTS and its association with joint biology

The synovial membrane is remodeled during the pathogenesis of OA, driven by pro-inflammatory cytokines; and by fragments known as DAMPs (damage associated molecular patterns) which are released from cartilage into the joint cavity upon trauma, and stimulate toll-like receptors which mediate further inflammation [28]. As synovial fibroblasts are activated, they proliferate and start secreting ECM proteins such as type III collagen. The synovial membrane thickens and become vascularized. OA pain is derived from many tissue components, but synovial vascularization and innervation are believed to be major contributors [21,29]. Increased expression and turnover of type III collagen, with associated release of COL3/ADAMTS from a thickening synovial membrane, may precede the onset of mild to moderate pain, when the tissue becomes innervated. Indeed, OA patients with no pain had higher levels of serum COL3/ADAMTS compared to those with mild to moderate pain. In addition, hSMEx COL3/ADAMTS release peaked with inflammatory stimulation early in the explant model at day 3, and decreased to W/O control levels by day 14. Further indicating that COL3/ADAMTS is a marker, which may flag the transition between asymptomatic OA and early progressive symptomatic OA.

ADAMTS expression has been shown to be elevated in synoviocytes and chondrocytes of OA patients [30,31], and plays a central role in the remodeling of joint connective tissues. Several markers and fragments of aggrecan resulting from ADAMTS activity have been described. Fragments containing the

sequences ARGS and NITEGE (e.g. measured by the AGNx1 ELISA) have been extensively investigated, and biomarkers measuring these neoepitopes have been developed [32,33]. Previously, ADAMTS activity was associated only with degradation and deterioration of the joint; however, recent data suggest that ADAMTS' play a vital role in overall tissue turnover. Elevated ADAMTS expression may therefore reflect an ongoing, potentially uncontrolled, repair process in response to inflammation and injury; and may explain the inverse relationship between COL3/ADAMTS and pain observed in this study.

4.3 The perspective

At present there no DMOAD available for OA, however drug are being developed which are believe to change joint metabolism. One of these treatments are targeting ADAMTS [34], which have traditionally been monitored by biomarkers reflecting cartilage degradation through the release of the neo-epitopes ARGS or TEGE from aggrecan [35]. COL3/ADAMTS could be used as a pharmacodynamics marker measuring the effect of anti-ADAMTS' on other tissues than cartilage.

4.4 Limitations.

There are several limitations in this study. Firstly, patients with systemic diseases other than musculoskeletal disease were not excluded from the study and results were not adjusted for NSAID use; both of which could influence serum biomarker levels. Secondly, the clinical study investigated a cross-sectional cohort, which focused on pain parameters alone, thus cannot provide insight in to the prognostic value of the biomarker.

5 CONCLUSION

In conclusion, the COL3/ADAMTS ELISA was technically robust and specific for the selected ADAMTS-1, -4-, -8 generated neoepitope. There was an inverse correlation between COL3/ADAMTS and pain scores, which was mainly associated with radiographic OA. These data may indicate that COL3/ADAMTS is a marker of early osteoarthritis. Such a biomarker could be used to characterize patients with early and mildly progressed disease, and may shed light on which degenerative components contribute to pain perception; since there is a disconnect between the degree of joint degeneration and joint pain.

6   ACKNOWLEDGEMENT

7   FORMATTING OF FUNDING SOURCES

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Table 1. The C4P-003 study overview

	1 (No pain) VAS pain <10 mm		2 (mild pain) VAS pain 10-40 mm		3 (moderate pain) VAS pain 40-70 mm		4 (severe pain) VAS pain 70-100 mm	
N	52		81		67		61	
No. of females (%)	23 (44)		46 (57)		35 (52)		34 (56)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Age (years)	67.5	4.8	62.8	8.3	64.7	7.6	63.0	8.1
BMI	27.2	4.0	27.5	3.8	27.9	3.3	29.5	4.9
Disease duration (years)	6.9	4.9	6.4	5.8	8.1	12.9	9.9	1.2
VAS pain score (mm)	1.1	2.3	26.2	9.3	54.3	8.1	78.7	7.0
Lequesne pain score	0.8	1.5	4.3	1.6	5.0	1.5	5.8	1.8
WOMAC_PAIN	1.0	2.0	6.6	2.8	8.1	2.5	9.4	3.1
WOMAC function	2.3	4.1	14.8	9.1	22.9	10.6	28.0	10.5
WOMAC stiffness	0.6	1.1	3.0	1.5	3.9	1.3	4.4	1.6
KLG, worst score	1.5	0.7	1.9	0.8	2.3	1.1	2.2	0.9

Table 2: COL3/ADAMTS ELISA technical performance. A: Measurement range**. B:** Linearity (percentage dilution recovery) of the COL3/ADAMTS ELISA using human serum samples from healthy donors (n=3). **C:** Inter- and intra-assay variation of the COL3/ADAMTS ELISA using human serum and urine QC samples; n=6 with 10 independent runs of each QC sample.

Technical performance of the COL3/ADAMTS ELISA						
A: Measurement range		**IC50, ng/mL**	**Slope**		**Quantification range, ng/mL**	
		13.7 [12.7 – 14.9]	0.99 [0.90 – 1.07]			2 - 60
B: Linearity	**Conc., ng/mL**	**% recovery**	**Conc., ng/mL**	**% recovery**	**Conc., ng/mL**	**% recovery**
Undiluted human serum sample	27.4	100.0	37.2	100.0	47.3	100.0
Dilution 1 in 2	14.0	102.4	17.9	96.3	24.9	105.3
Dilution 1 in 4	7.3	106.8	8.4	90.6	13.1	110.4
Dilution 1 in 8	3.6	105.5	4.5	96.4	6.0	101.6
Dilution 1 in 16	2.0	*118.6*	2.6	112.1	3.7	*124.3*
Average recovery, %		**99.1**		**106.6**		**108.3**
C: Assay Variability						
Precision	**ng/mL**		**Intra-assay variability %**		**Inter-assay variability %**	
Sample 1		31.6		5.5		7.5
Sample 2		50.3		3.6		3.1
Sample 3		60.1		5.7		3.0
Sample 4		69.2		7.0		5.1
Sample 5		64.3		3.9		5.6
Sample 6		80.6		6.0		5.9
Mean				**5.3**		**5.0**

Table 3. Correlation between COL3/ADAMTS and clinical pain and function measures.

	COL3-ADAMTS		COL3/ADAMTS adjusted for age, gender, BMI and smoker status		
	R partial	*P value*	*R partial*	*P value*	*Significant covariates*
VAS PAIN	-0.14	0.020	-0.15	0.018	BMI
WOMAC PAIN	-0.16	0.010	-0.17	0.0065	BMI
Lequesne Pain	-0.17	0.0070	-0.13	0.035	BMI, Age, Gender
WOMAC function	-0.10	0.099	-0.11	0.066	BMI
WOMAC stiffness	-0.08	Ns	-	-	BMI, gender

Table 4. Levels of serum COL3/ADAMTS in patients with different knee OA pain and radiographic phenotypes. The data was corrected for age, gender, BMI and smoker status.

	1 (No pain) VAS pain <10 mm			2 (mild pain) VAS pain 10-40 mm				3 (moderate pain) VAS pain 40-70 mm				4 (severe pain) VAS pain 70-100 mm			
	N	Mean	95% CI	N	Mean	95% CI	P	N	Mean	95% CI		N	Mean	95% CI	
All	52	31.1	25.9 to 36.3	81	19.2	14.4 to 24.0	0.0076	67	17.0	11.5 to 22.6	0.0019	61	24.9	17.7 to 32.1	Ns
NON RKOA	25	30.7	23.2 to 38.2	21	20.7	12.3 to 29.0	Ns	14	13.6	3.7 to 23.5	Ns	8	29.4	15.9 to 42.9	Ns
RKOA	27	31.5	24.4 to 38.7	60	17.7	12.9 to 22.4	0.0086	53	20.5	15.4 to 25.5	0.080	53	20.4	15.3 to 25.6	0.097

Table 5. Asymptomatic RKOA vs. symptomatic RKOA. The comparisons were done on Z-transformed marker levels.

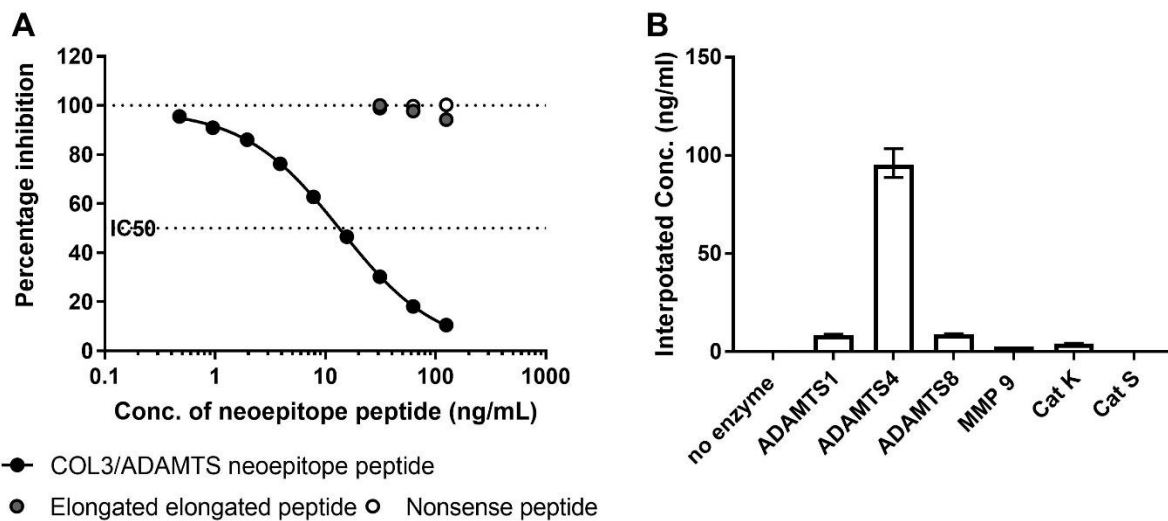|	The marker			The model			
	OR	95% CI	P value	AUC	95% CI	P value	Covariate
COL3-ADAMTS	0.56	0.41 to 0.77	0.0003	0.72	0.66 to 0.71	<0.0001	Age
C3M	0.40	0.87 to 0.97	<0.0001	0.78	0.72 to 0.83	<0.0001	Age, BMI
AGNx1	1.84	0.96 to 3.53	0.066	0.65	0.59 to 0.71	0.024	Age
Age	0.92	0.88 to 0.97	0.0010	0.67	0.61 to 0.73	0.0003	-
BMI	1.08	0.99 to 1.16	0.093	0.59	0.53 to 0.65	0.083	-

Figure 1. **Characterization of the COL3/ADAMTS mAb; specificity of type III collagen neoepitope recognition. A)** COL3/ADAMTS ELISA standard curve and epitope specificity test. Four-parameter interpolated curve using the specific peptide, as well as test of the elongated and a nonsense peptide. **B)** COL3/ADAMTS ELISA assessment of in vitro cleaved type III collagen by common proteases of the ECM. Each bar contains four replicates and are shown as mean with SEM bars.
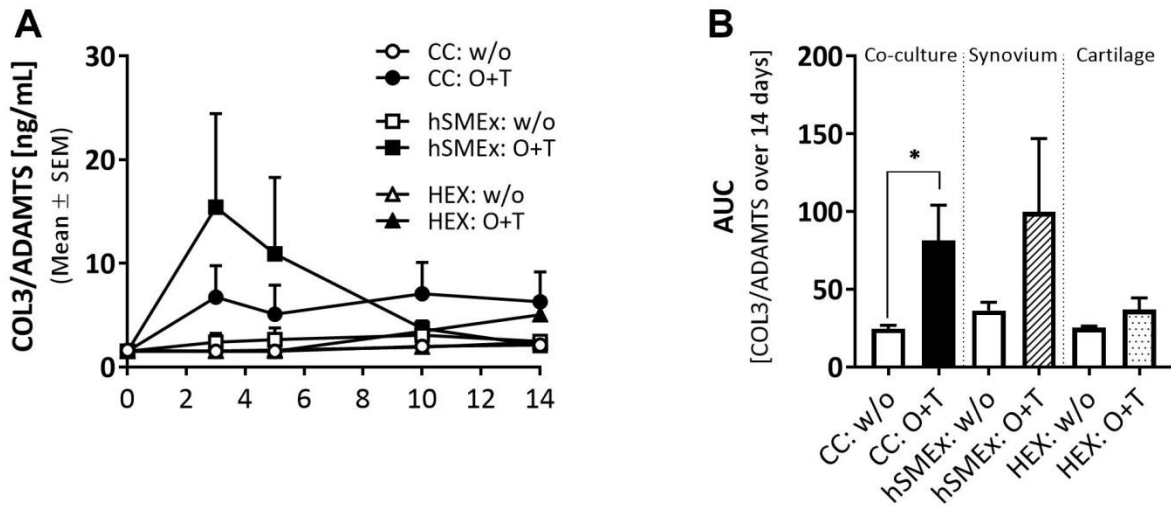
Figure 2. Biological target validation using the COL3/ADAMTS ELISA. A) Time-depended release of COL3/ADAMTS from synovial membrane (hSMEx) or cartilage explants (HEX) alone or in co-cultures (CC) in the presence or absence of the pro-inflammatory cytokines oncostatin M and TNF alpha (O+T). Tissue from four different patients was used with six replica per condition. **B)** Accumulated release of COL3/ADAMTS from the explant cultures after 14 days. Error bars are SEM. * indicates statistical significance (p<0.05).