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Blood and urine biomarkers in osteoarthritis – an update on cartilage associated type II collagen and aggrecan markers

CURRENT OPINION

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Purpose of review
Osteoarthritis (OA) is a painful disease for which drug development has proven difficult. One major reason for this is the heterogeneity of the disease and the current lack of operationalized means to distinguish various disease endotypes (molecular subtypes). Biomarkers measured in blood or urine, reflecting joint tissue turnover, have been developed and tested during the last decades. In this narrative review, we provide highlights on biomarkers derived from the two most studied and abundant cartilage proteins – type II collagen and aggrecan.

Recent findings
Multiple biomarkers assessing type II collagen degradation and formation, and aggrecan turnover have been developed. Several markers, such as uCTX-II, have been validated for their association with disease severity and prognosis, as well as pharmacodynamically used to describe the mode of action and efficacy of drugs in development. There is a great need for biomarkers for subdividing patients (i.e., endotyping) and recent scientific advances have not yet come closer to achieving this goal.

Summary
There is strong support for using biomarkers for understanding OA, reflecting degradation and formation of the joint tissues, focused on type II collagen and aggrecan. There is still a lack of in vitro diagnostics, in all contexts of use.

Keywords
aggrecan, biomarker, osteoarthritis, type II collagen

INTRODUCTION

The most prevalent form of arthritis is osteoarthritis (OA). It is associated with overuse of the joints, injury, metabolic and inflammatory factors, and accounts for more than 10% of disabilities and symptomatic diseases of the elderly population [1,2]. Joint pain and progressive degeneration of articular cartilage are hallmarks of the disease which include remodeling of all joint tissues (bone, synovium, ligaments) resulting in painful articular cartilage loss, manifested as radiographic joint space narrowing (JSN) [3]. Research indicates that the structural integrity and preservation of articular cartilage is highly reliant on normal subchondral bone turnover, synovial crosstalk, physiological biomechanical stresses and intact chondrocyte function [4]. Both in the clinical and research settings, and possibly most importantly in drug development, there is a need for validated diagnostic tools for treatment selection. Thus, developing markers

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KEY POINTS

- Biomarkers of cartilage proteins type II collagen and aggrecan are the most abundant and most studied to date.

- Several biomarkers have been developed that target breakdown or formation peptides of type II collagen and aggrecan.

- Many of the existing OA-related biomarkers are associated with radiographic severity, progression, and pharmacodynamics.

- Very few biomarkers have been tested in the context of identifying specific endotypes of OA.

with these properties is a top priority for researchers in the biomarker field for future management of OA.

Disease-modifying OA drugs (DMOADs), i.e., drugs that could ease pain and slow the destruction of joints, have not yet been approved for OA [5,6]. The lack of biological understanding what drives OA and a heterogeneous patient population may explain why no medication has thus far been developed. Therefore, we need to expand our understanding of the subclinical or molecular subtypes, i.e., endotypes, as well as the corresponding markers that allow separation of patient groups.

Although many aspects of biomarker development need attention, we have elected to focus on the following: (1) Pharmacodynamic biomarkers allowing the monitoring of treatment responses within a clinical study enabling informed decision making in clinical studies faster and with fewer patients, analogous to the response of HbA1c in type II diabetes studies. (2) Biomarkers reflecting disease activity, which may possibly be used for identification of fast progressors and those in most need of treatment. This will also allow for selection of the optimal patients for clinical studies.

Blood, synovial fluid, or urine-based biochemical markers may be used as noninvasive and objective measures to diagnose patients. In these settings, there are a range of confounding factors associated with noninvasive biochemical markers which need to be discussed and considered, to provide the right context for evaluating the potential value of biomarkers [7].

This narrative review sets out to review current and potential biomarkers for use in OA with focus on cartilage-derived type II collagen and aggrecan markers. We have searched PubMed for recent papers and selected a few to highlight as examples of the research conducted to develop and test biomarkers for OA.

## OSTEOARTHRITIS – THE STORY IS EVOLVING

A long list of biomarkers has been tested in OA for different purposes and listed in several recent reviews [7–9]. For the purpose of a concise description, we will only describe type II collagen and aggrecan, which are the main components of cartilage although other proteins may be of equal interest.

Type II collagen – keeping it together

Type II collagen is the most abundant protein in cartilage [10]. It is a fibrillar collagen that acts as the structural framework of the cartilage extracellular matrix (ECM). Collagen attracts attention as its turnover is directly associated with cartilage homeostasis and joint health. Several markers have been developed targeting different soluble metabolites and posttranslational modifications of type II collagen (Fig. 1).

Formation of type II collagen

As the collagen matures both the N- and C-terminal propeptides are cleaved off (Fig. 1). Alternative splicing of the first three exons results in two types of the noncollagenous N-terminal pro-peptide—types IIA and IIB. Type IIA is the embryonic variant in which the cysteine-rich region, exon 2, is retained, whereas type IIB is the variant present in mature articular cartilage [11]. Markers that measure either of the two splice variants have been developed. The PIIANP assay targets the A form of the splice variant [12]. In the OA biomarker project, Foundation for the National Institutes of Health (FNIH, US) study, baseline serum levels of PIIANP were found to be negatively associated with two-year radiographic progression when assessed in 600 OA patients [13]. An immuno-assay specific for PIIBNP was recently developed [14,15]. The PRO-C2 assay, targeting an epitope at the junction site of exons 1 and 3, only measures released PIIBNP. Compared with healthy controls ($n = 22$), PRO-C2 and PIIANP in serum samples were lower in knee OA patients with a KL grade of 2–4 ($n = 123$) [15]. Assessed two independent knee OA cohorts of 106 and 147 participants, Luo *et al.* reported that low serum levels of PRO-C2 were associated with 3.4-fold higher likelihood of radiographic progression than those with high levels of PRO-C2 [16■]. This suggests that low cartilage repair capacity, assessed by markers such as PRO-C2 and PIIANP, is a risk factor for OA. The idea of low cartilage formation being associated with OA severity and progression was already proposed two decades ago by Garnero *et al.*, who showed that the ratio between type II collagen synthesis measured by PIIANP and degradation measured by uCTX-II was
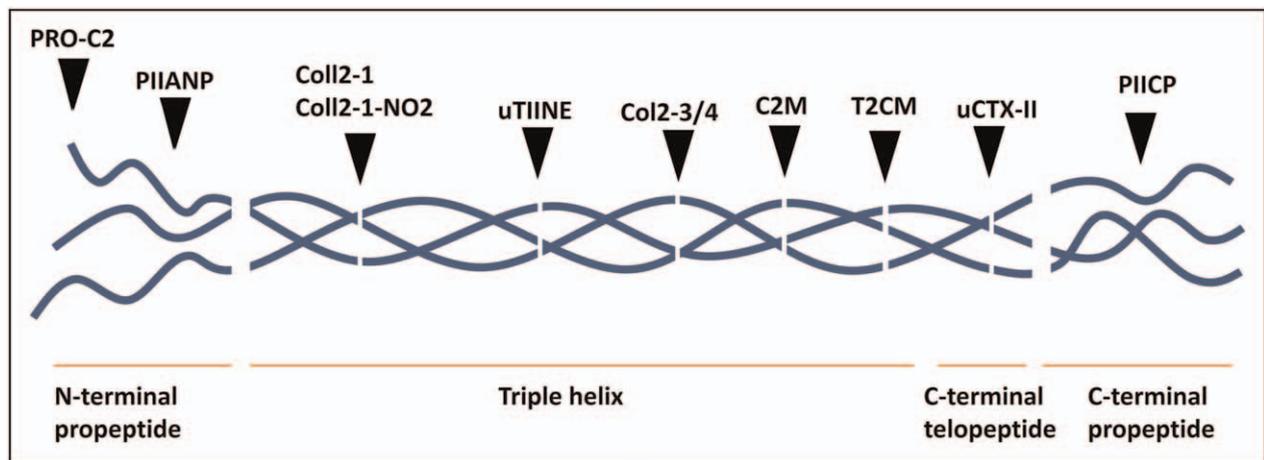
FIGURE 1. Type II collagen and its biomarkers. Type II collagen is a fibrillar collagen composed of three alpha 1 chains. It has been targeted extensively for biomarker development as it is cleaved at multiple locations in the triple helical and telopeptide regions. Type II collagen has N- and C-terminal propeptides, which are cleaved off at maturation; their measurement reflects cartilage formation.

altered in OA [17]. Moreover, these data are now appreciated as indications of different endotypes in OA. PIICP has also been suggested to be a marker of cartilage formation, however to date, it has mainly been assessed in preclinical settings and posttraumatic injury cohorts.

Degradation of type II collagen

The N- and C-telopeptide, of which the C-terminal telopeptide contains the lysine residue used for inter-crosslinking, are still present on the mature secreted and cartilage-incorporated type II collagen (Fig. 1) [18]. Matrix metalloproteinases (MMPs), including collagenases and gelatinases, are the main 'actors' in type II collagen degradation. In particular, MMP-1, -8, and -13 cleave the site between glycine775 and leucine776, the primary cleavage site in the collagen triple helix, resulting in the unwinding of the helix. This cleavage generates two new peptides that are 3/4 and 1/4 the length of the original mature type II collagen. Both the primary cleavage and the further secondary processing of these domains produce fragments that are believed to be released to circulation and can be targeted via their newly generated neoepitopes [19].

Billinghurst *et al.* generated the C1,2C (also denoted as COL2-3/4Cshort) assay, which targets a neoepitope located at the C-terminus of the collagenase-generated 3/4 fragment [20]. Quantification of C1,2C in cartilage from OA patients and healthy controls in which type II collagen has been extracted showed an increase in C1,2C levels in OA cartilage compared to the healthy samples [20]. However, the C1,2C assay is limited by cross-reactivity toward type I collagen (''the bone collagen''). To address this cross-reactivity, a sandwich ELISA, uTIINE, was

developed that targets a type II collagen-specific epitope upstream of the 3/4 cleavage sites. UTIINE was detected in 9/10 OA patients but only in 2/10 of healthy controls [21], indicating diagnostic potential for the assay. Since the development of the assay, the cleavage was found to be mainly mediated by MMP-13 activity [21]. uTIINE was able to distinguish between OA patients and healthy controls, as well as between symptomatic and asymptomatic radiographic OA patients [21]. Furthermore, in a randomized controlled trial testing doxycycline treatment in 120 obese knee OA patients, increased uTIINE levels were associated with increased JSN [22].

Another collagen type II assay, the C2C assay, quantifies an elongated version of the C1,2C target sequence, which—in contrast to C1,2C—is specific toward type II collagen. There have been several recent reports indicating that C2C measured in serum or urine (C2C-HUSA) is associated with radiographic OA progression [13]. Poole *et al.* reported that urinary C2C was significantly higher in progressors compared to nonprogressors after a 3-year follow-up period [23]. In the OAI-FNIH study only urinary C2C (C2C-HUSA) but not serum C2C was associated with knee OA progression [13].

The biomarker Coll2-1 targeting an epitope located in the N-terminal triple helical region of the 3/4 fragment is another cartilage degradation marker being tested in OA. The targeted epitope contains a tyrosine residue that is susceptible to nitration, resulting in the release of the neo-epitope Coll2-1-NO2. Increased levels of oxidative and inflammatory stress have been detected in OA and rheumatoid arthritis, suggesting that posttranslational species of ECM markers, such as Coll2-1-NO2, could provide more specificity to the markers

for reflecting molecular pathways [24]. Further investigations of this association were performed in a follow-up study, in which an increase in urinary Coll2-1 levels was recently shown to be correlated to subscores of Whole-Organ Magnetic Resonance Imaging Score (WORMS), amongst other articular cartilage integrity and osteophyte scores [25▪]. In multivariate analysis of the 19 available markers measured in the FNIH cohort, Liem *et al.* reported that Coll2-1-NO2 together with three other markers (CS846, COMP and uCTX-II), were independently associated with radiographic OA severity [26].

The C2M biomarker was identified by mass spectrometric analysis of the fragments generated by MMP-9 cleavage of healthy human cartilage [27]. C2M reflects a neoepitope located in the C-terminus of the 1/4 fragment close to the C-terminal domain [28]. Serum C2M has been reported to be higher in patients with radiographic knee OA compared with healthy controls. However, recent reports indicate that this marker is highly elevated in rheumatoid arthritis and ankylosis spondylitis, rather than OA, thus it may find its home in that corner of rheumatology.

uCTX-II is probably the most well-described biomarker of type II collagen [29]. Measurements of urinary uCTX-II were able to distinguish between OA patients and healthy controls and between OA patients with slowly and rapidly progressing disease [17,29]. Kraus *et al.* showed that baseline uCTX-II was able to predict both symptomatic and radiographic knee OA progression [13]. These findings were confirmed by a recent meta-analysis by Huan *et al.*, which concluded that uCTX-II levels were higher in OA patients than in healthy controls and that uCTX-II levels increased with disease progression [76]. A very recent metaanalysis by Cheng *et al.* summarized the value of uCTX-II. By pooling data from 2856 participants, they found uCTX-II to be higher in patient with severe OA compared to patients with moderate OA [30▪]. Interestingly, they also found that uCTX-II performed better in females vs. males, and in European vs. Asian populations. This highlights the importance of including variables related to human diversity, such as sex and race, at a minimum, as covariates in evaluations of the diagnostic value of novel biomarkers. In addition, uCTX-II has been applied in DMOAD trials, as demonstrated by Manicourt *et al.* wherein change in uCTX-II levels correlated with the treatment-mediated change in disease status; and as demonstrated by Karsdal *et al.* and Bihlet *et al.*, examining the effect of oral salmon calcitonin (sCT) on Lequesne algofunctional index scores in patients with knee OA and its association with pain and radiographic outcomes [31–33]. There have been several attempts to develop a serum CTX-II assay, however with limited success. T2CM is a recently discovered serum marker targeting a neoepitope generated by MMP-13 by cleavage of the C-terminal end of the triple helix close to the telopeptide end [34]. The first results show that T2CM is increased in patients scheduled for joint replacement compared to patients with moderate OA. In addition, the levels of T2CM were significantly decreased in response to sCT compared to placebo [34].

AGGRECAN – A STORY OF ITS OWN

Aggrecan is the main ECM glycoprotein of articular cartilage (Fig. 2) [35]. Aggrecan is a protein that has been modified with large polyanionic carbohydrates, primarily chondroitin sulfate. It binds to hyaluronic acid and link proteins [36] and draws cations and osmotically obliged water into the ECM; this creates a hydrated gel, that gives the cartilage its compressive strength. Aggrecan is important for the physiochemistry and biology of the cartilage, it is
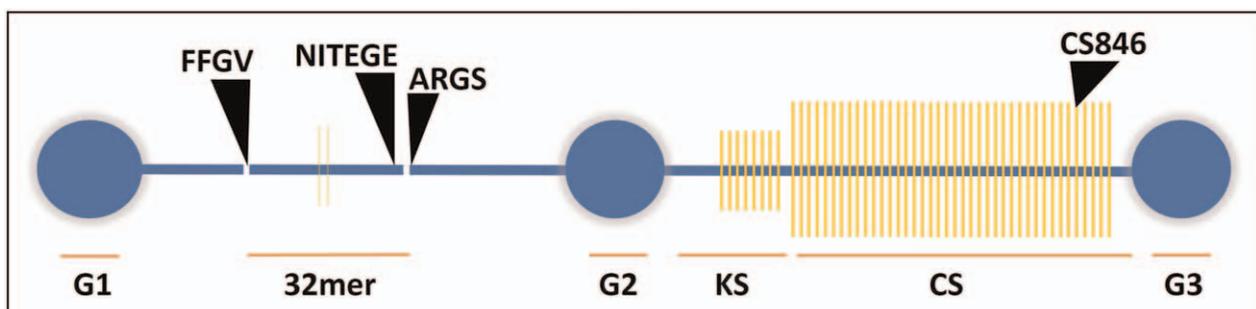
FIGURE 2. Aggrecan and its biomarkers. Aggrecan consists of three globular domains – G1-3, and two interglobular domains. The C-terminal interglobular domain contains keratan sulfate (KS) and chondroitin sulfate (CS) rich areas. MMP and ADAMTS cleavage fragments released from the N-terminal interglobular domain have been targeted for biomarker development (FFGV generated by MMPs, and NITEGE and ARGS by ADAMTS). The 32mer fragment from FFGV to NITEGE has been reported to have signaling functions. CS846 has been proposed to be a marker of aggrecan formation.

not just a structural glycoprotein. Three globular domains (G1, G1 and G3) make up aggrecan together with carbohydrate recognition domains, epidermal growth factor-like domains, complement binding protein-like domains, immunoglobulin folds and proteoglycan tandem repeats in the inter-globular domains [37] (see Fig. 2 highlighting the most well-described aggrecan cleavage sites by A Disintegrin And Metalloproteinase with Thrombo-spondin motifs (ADAMTS') and MMPs).

There is disagreement regarding the relationship of the two aggrecanases, ADAMTS-4 and ADAMTS-5, with respect to how they fit into the pathology of OA, loss of cartilage and aggrecan. Bondeson *et al.* [38] have highlighted that in murine OA, induced by antigen or surgical joint destabilization, ADAMTS4-null mice had no protective effect on cartilage aggrecan loss in contrast to ADAMTS5-null mice. ADAMTS-5 and ADAMTS-4 mRNA are expressed by freshly isolated human OA synovial cells and ADAMTS-5 by freshly isolated human OA chondrocytes [39]. Both ADAMTS-4 and -5 cleave aggrecan but they are different enzymes regarding their regulation.

Although insoluble type II collagen turns over slowly after skeletal maturity, aggrecan is very regenerative, thus its degradation is in theory, reversible [40]. Heinemeier *et al.* [41] elegantly dem-onstrated that bomb pulse ^{14}C levels in insoluble type II collagen from articular cartilage remained nearly unchanged from the age of 13 and found that OA seemed to have practically zero impact on the turnover of insoluble type II collagen after this age. ^{14}C levels in the proteoglycan component of carti-lage were practically like atmospheric ^{14}C levels during biopsy extraction. This important finding indicated that preservation, healing, and modifica-tion of cartilage tissue is primarily possible for the proteoglycan component but not the insoluble col-lagen component in skeletally mature individuals.

Within OA, aggrecan destruction has impor-tance and is an important structural protein in the cartilage ECM. Fosang *et al.* have described aggrecan mutations in mice that prohibited carti-lage degradation with only a bit of loss of collagen [42]. Recent data by Reker *et al.* [43] showed that fibroblast growth factor 18 induced a biphasic remodeling process in OA cartilage explants charac-terized by an increase in aggrecanase degradation of aggrecan reflected by the biomarker AGNx-1 (NITEGE, Fig. 2) paralleling the initial decrease in type II collagen formation marker PRO-C2 in the first three weeks of culture. This was followed by a decrease in AGNx-I and an increase in PRO-C2 the remaining 7 weeks of culturing [43]. Thus, the ana-bolic process of cartilage aggrecan restructuring is thus very dynamic. A direct causal relationship has also been suggested between aggrecan destruction, binding of aggrecan fragments to the toll-like recep-tor-2, IL-6 induction, and pain [44], which could provide a temporal link between disease associated tissue activity and OA symptoms

Assessing A Disintegrin And Metalloproteinase with Thrombospondin activity by quantifying the ARGS neo-epitope

Degradation of aggrecan

The degradome profile of aggrecan is extremely complex, but this is possibly one of the most inves-tigated ECM glycoproteins despite the complexity. A range of biomarkers have been developed for the assessment of aggrecan destruction (Fig. 2). The aggrecan inter-globular domain displays several cleavage sites susceptible to MMPs, ADAMTS', ser-ine, and cysteine proteases [45]. Multiple protease cleavage sites have been found in the aggrecan G1-G2 domain [35]. These different cleavage sites give rise to neo-epitopes, for example, FFGV (AGNx-2), NITEGE (AGNx-1) and ARGS, which have been tar-geted as biomarkers of cartilage turnover. Although markers assessing FFGV and NITEGE seem to be stuck in the preclinical space, ARGS has been mea-sured in several clinical studies during the last three decades. Below we will discuss some of the more recent studies. There are also several cleavage sites in the G2-G3 domain, however, these targets are less well-described in the literature, potentially due to the inaccessible nature of the highly glycosylated domain.

The marker ARGS released from aggrecan because of protein cleavage by ADAMTS' and several assays are available measuring the fragment in serum or plasma. It is generally considered a marker of cartilage degradation although, as discussed above and shown by Reker *et al.*, it could also be a marker of cartilage remodeling. Consequently, although generation of the ARGS cleavage site is a direct consequence of ADAMTS activity, interpre-tation of protease cleavage sites and the relevant pharmacology need to be carefully evaluated. An alternate cleavage pattern by compensatory pro-teases is sometimes induced when a protein cannot be remodeled by one protease, resulting in an alter-native cleavage pattern, such as the MMP cleavage of aggrecan. A similar observation, related to type I collagen, has already been made during the clinical development of Cathepsin K inhibitors for osteo-porosis. CTX-I is a bone resorption biomarker generated by Cathepsin K activity on type I collagen [46]; Cathepsin K inhibition reduced the

pharmacodynamic biomarker, CTX-I, more than 90%, whereas another biomarker of type I collagen destruction by MMPs, ICTP, was elevated. Thus, in addition to ADAMTS-5, ADAMTS-4, other proteases may be involved in pathological turnover of aggrecan thereby requiring a multiprotease inhibition strategy to successfully slow OA based on this target. Alternative biomarkers of type II collagen and other fragments of aggrecan destruction likely need to be investigated in the context of any aggrecanase inhibition trial to lower this risk.

Formation of aggrecan

Unlike type II collagen, aggrecan does not have propeptides which are released upon maturation of the protein. CS846 has been proposed as a marker of aggrecan formation, although the original literature described this epitope as a marker of aggrecan turnover [47]. CS846 was amongst four of 19 biomarkers tested in the OAI-FNIH cohort that was independent associated with radiographic OA [26]. CS846 was decreased modestly in response to collagen hydrolysate compared to controls, which contrasted with PIIANP which was elevated [48]. These data may indicate a disconnect between aggrecan and type II collagen formation.

ON A SIDE NOTE

Type II collagen and aggrecan are the not the only proteins for which tissue associated markers have been developed and tested during the last decades. It is worth mentioning markers such as COMP, measuring the turnover of the cartilage oligomeric matrix protein in blood. It seem to be highly elevated after traumatic injury and moderate to high with impact exercise [13,49,50]. CRPM, which measures a metabolite of C-reactive protein, is also elevated in a subset of OA patients and associated with disease progression [51–53]. In addition, several markers reflecting bone and synovial turnover and inflammation have been and are currently being tested in wide range of OA cohorts. Each of these deserve a review of its own.

CONCLUSION

Type II collagen and aggrecan are likely the most studied cartilage proteins with respect to developing biomarkers. Some biomarkers have been tested for their association with disease severity, others for their prognostic and pharmacodynamic ability. There is no doubt that many of the markers are good for understanding biology, however, there is a lack of markers that have reached the level of *IVD* use. Only a few of the markers discussed here have been tested in

the context of identifying OA endotypes. This may be a field where serum and urine markers have the ability to provide groundbreaking value.

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Conflicts of interest

A.C.B.J., C.S.T. and M.A.K. are full-time employees and shareholders of Nordic Bioscience, a privately owned biotechnology company involved in the development of biomarkers for OA. There are no conflicts of interests for the remaining authors.

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