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PERSPECTIVES

New Biochemical Markers of Cartilage Turnover in Osteoarthritis: Recent Developments and Remaining Challenges

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Abstract

Osteoarthritis (OA) is characterized by progressive destruction of articular cartilage and by subchondral bone and synovial tissue reaction. Radiological findings are not sufficiently sensitive to detect early disease or to monitor the progression of joint damage. This article reviews recent developments in biochemical marker assays of cartilage turnover and explores their potential clinical uses in OA. Because type II collagen (CII) and aggrecan are the most abundant proteins in the cartilage matrix, efforts are centered on identifying biochemical markers of their synthesis and degradation. Assays for the N-terminal propeptides of procollagen IIA (PIIANP) and IIB (PIIBNP) have been developed to measure type II collagen synthesis. Degradation can be monitored by analyzing fragments from the helical (Helix-II, Col 2-1) or C-telopeptide (CTX-II) regions. Antibodies directed against the chief proteolytic cleavage sites of aggrecan by metalloproteases and aggrecanases have been generated and used in different assay formats. Although these new aggrecan markers have been useful in investigations of the biological pathways of cartilage turnover in *in vitro* and animal models, few of them have yet been carefully evaluated in patients with OA. Conversely, prospective studies suggest that systemic levels of some markers of type II collagen degradation (e.g., Helix-II and CTX-II) and synthesis (PIIANP) can predict disease progression in knee and hip OA, particularly when used in combination. An optimal panel of markers of cartilage, synovial tissue, and bone will certainly play an important role in the clinical development of new disease-modifying therapies and ultimately in the management of OA. *BoneKEy-Osteovision*. 2007 January;4(1):7-18.

OA is a prevalent, age-related disease characterized by degraded cartilage, mild to moderate synovial inflammation, and altered bone structure; it results in pain, impaired mobility, and disability. Plain radiography, the reference technique for assessing the severity of joint destruction, provides direct information on bones but not on cartilage, and has limited sensitivity. Magnetic resonance imaging (MRI) provides direct information on the different joint structures and is currently being optimized for use in OA. Although twin studies have clearly demonstrated a strong genetic component of OA, the gene(s) of most importance in assessing disease progression remain to be determined. In addition, genetic polymorphisms are likely to be of limited value in assessing the effects of therapies targeting prevention of joint damage. Conventional laboratory tests, such as serum C-reactive protein, show inflammation in some patients but provide little information on joint damage. These limitations have led to considerable interest in exploring the potential of specific biological markers that reflect dynamic variations in joint-tissue remodeling, as recently reviewed (1) (see Table 1).

New Markers of Cartilage Metabolism

Biochemical markers for OA research and patient management can be conveniently classified according to the BIPED terminology (Burden of the disease, Investigative, Prognosis, Efficacy of intervention, and Diagnosis), which has been recently proposed by the NIH Osteoarthritis Biomarkers Network (2). To fulfill the specifications of this classification, biochemical markers need to meet the criteria listed in Table 2.

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Because of the focal nature of OA, the small amount of cartilage in the body, and the involvement of different tissues (e.g., bone,

	Synthesis	Degradation
Cartilage		
Type II collagen	●N- and C-propeptides (PIICP, PIIANP, PIIBNP, total PIINP)	● PYD ●Type II collagen C-telopeptide (CTX-II) ●Type II collagen collagenase neoepitope (C2C C12C, TIINE) ●Type II collagen helical fragments (Helix-II and Coll 2-1, Coll 2-1 NO2)
Aggrecan	●Chondroitin sulfate (epitopes 846, 3B3, 7D4) ● G1/G2 domain	●Core protein MMPs and aggrecanase neoepitopes ● Keratan sulfate (epitopes 5D4, ANP9)
Non-aggrecan and non-collagen proteins	● Glycoprotein 39 (YKL-40) ● Cartilage-derived retinoic acid sensitive protein (CD-RAP)	● COMP
Synovium/synovitis Type I/III collagen	● Type III N-propeptide (PIIINP)	● PYD ● CTX-I, NTX-I ●Glucosyl-galactosyl-pyridinoline (Glc-Gal-PYD) ●Nitrosylated type III collagen N-telopeptide (IIINys)
Non-Collagenous proteins	● Hyaluronan ● YKL-40 ● COMP	
Proteases	● MMP-1, -2, -3, -9	
Systemic inflammation	Ultrasensitive C-reactive protein (CRP)	

Table 1: Candidate biological markers of cartilage and synovial tissue metabolism

Factor	Comment
Origin	- Joint (knee, hip, hand, spine…) - Tissue specificity: e.g., cartilage vs non-cartilage tissues - Spatial tissue distribution: e.g., superficial, pericellular, inter-territorial, deep, calcified zones of cartilage
Metabolism and clearance	- Mechanisms of release from the tissue: e.g., enzymatic pathways - Clearance from the joint cavity to circulation, synovial lymphatic breakdown, liver uptake, renal excretion, circulating half life
Assay features	- Circulating/urinary forms detected - Robustness (sample stability, analytical accuracy and precision) - Diurnal, short- and long-term intra-patient reproducibility - Clinical sensitivity and specificity - Accessibility, convenience, cost

Table 2: Criteria for biochemical markers of osteoarthritis

cartilage, and synovium) in the pathophysiology of the disease, one of the most important features of any biochemical marker is tissue specificity. Articular cartilage is a nonvascular tissue comprising two major phases: a fluid phase of water and electrolytes, and a solid phase composed of collagens, proteoglycans, glycoproteins, other proteins, and chondrocytes. CII provides by far the major

portion of the organic components (15%–22%), followed by aggrecan (4%–7%), a large proteoglycan, and other noncollagenous proteins (0.5%–1%), including cartilage oligomeric matrix protein (3). Because of their contribution to and specificity for cartilage matrix, the biochemical markers of the synthesis and degradation of CII and aggrecan are currently under extensive investigation. CII is synthesized by chondrocytes as a precursor, procollagen, which consists of CII itself, forming the framework of cartilage matrix, and N- (PIINP) and C-terminal (PIICP) propeptides at each end. The propeptides are cleaved off during the maturation of collagen molecules and are released into biological fluids; their concentration is believed to directly reflect the rate of CII synthesis. There are two alternative forms of type II procollagen, which differ by the presence (IIA) or absence (IIB) of a 69 amino acid sequence coded by exon 2 in the N-propeptide. PIIANP is expressed mainly during development, whereas the IIB variant is the major form in healthy adult cartilage. The C-propeptide (PIICP), which is common to PIIANP and PIIBNP, provides a global index of CII synthesis. Because CIIA can be re-expressed in OA cartilage (4), we were interested in developing an immunoassay that identifies PIIANP specifically by using an antibody raised against recombinant exon 2 (5). Compared to healthy sex- and age-matched controls, we found increased serum levels of PIIANP in early knee OA (6), whereas decreased values (7) were found in patients with advanced knee OA, suggesting that a cartilage repair mechanism could be effective in early OA, but may become deficient with advancing disease.

More recently, Olsen *et al*. (8) developed antibodies directed against a synthetic peptide of 15 amino acids, PIINP-I, which is common to both IIA and IIB N-propeptides. This new marker was found to be elevated in the supernatant of bovine cartilage explants activated by insulin growth factor-1, a strong anabolic agent. In patients with advanced rheumatoid arthritis (RA), plasma concentrations of PIINP-I were significantly lower compared to healthy controls, suggesting a deficient cartilage repair mechanism. Because assays for PIICP, PIIANP, and PIINP-I likely reflect different biological aspects of CII synthesis, studies comparing the levels of these three markers in patients with arthritis would be very useful in evaluating their respective value alone and in combination.

The recent development of assays for CII breakdown represents a breakthrough in the field of biological markers for OA, given that degradation of collagen fibers is associated with irreversible cartilage destruction. These assays are based on antibody detection of different proteolytic fragments of CII (Figure 1). The initial cleavage of CII molecules by collagenases 1, 2, and 3, and by membrane type 1-metalloproteases (MMPs), occurs between amino acids Gly^{794} and Leu^{795}, located one-quarter the length of the molecule from the C-terminal end, and can be monitored by various antibodies raised against the neoepitopes generated by this process (Figure 1). More recently, immunoassays based on antibodies raised against synthetic peptides derived from Helix-II or CTX-II have been developed. An important step in the validation of these new markers (Table 2) has been to dissect out the enzymatic pathways involved in their release. Indeed, it may be possible that different proteolytic mechanisms of cartilage degradation are involved at different stages of the disease, and in order to monitor the effects of anti-protease drugs – a treatment strategy currently under investigation in OA – knowledge of this timing is critical for a correct interpretation of biomarker data. Using a variety of *in vitro* and *ex vivo* models of bovine or human cartilage explants, we and others have shown that the fragments Helix-II and CTX-II are primarily released from different enzymatic pathways, cysteine proteases for Helix-II and MMPs for CTX-II (9;10). Critically important is the spatial distribution of these fragments within the cartilage tissue (Table 2). Immunohistochemistry of cartilage from ovariectomized rats (an animal model of mild cartilage erosion) or from patients with knee OA revealed that, although both Helix-II and CTX-II are localized at the surface of damaged cartilage, only CTX-II is also abundantly present at the bone-cartilage interface region within the calcified area (11;

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12). These findings may provide new insights into the pathophysiology of OA but could also have clinical applications. For example, we have shown that measurement of both Helix-II and CTX-II was more effective than either marker alone in

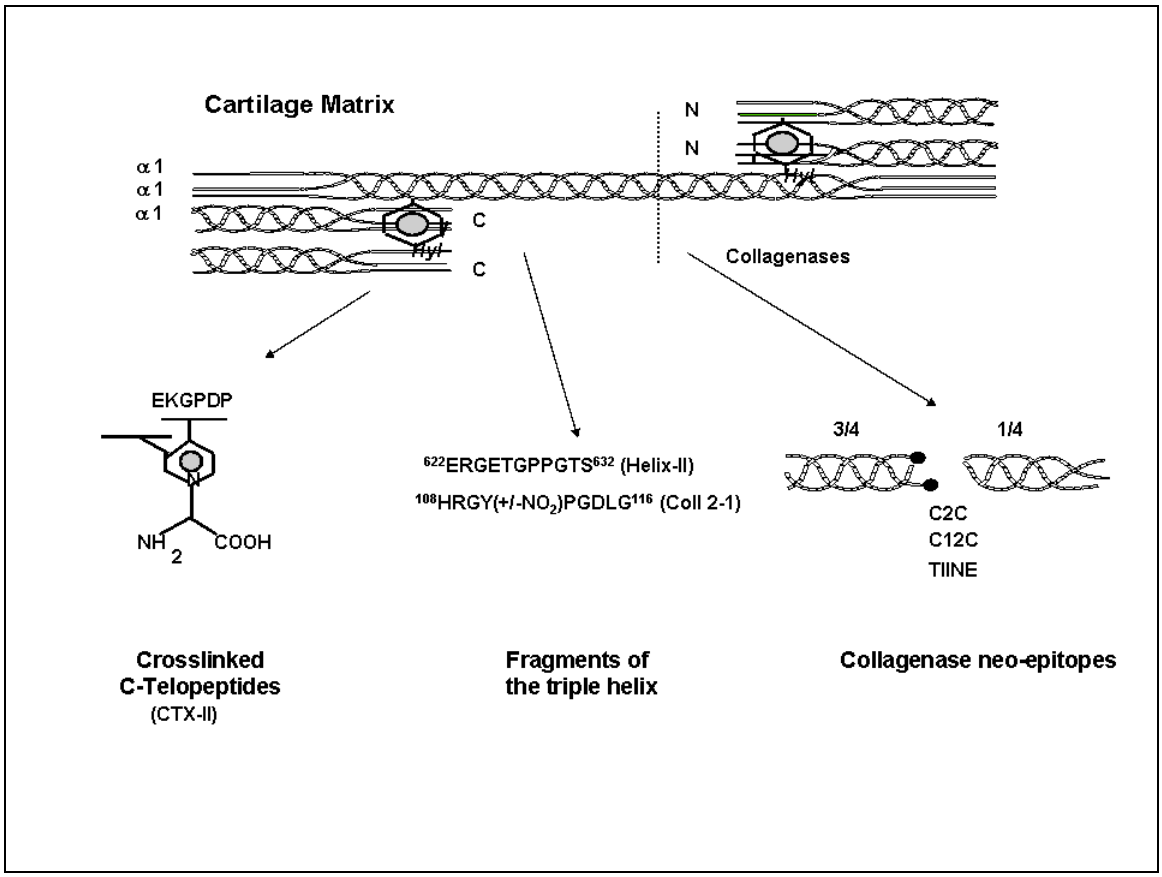

Figure 1: Type II collagen fragments as specific biological markers of cartilage degradation. Type II collagen is formed by the association of 3 identical α1 chains in a triple helix except at the ends (telopeptides). In the extracellular matrix of cartilage, collagen molecules are cross-linked by pyridinoline (PYD) in the telopeptide regions. During cartilage degradation, different molecules are released into synovial fluid, serum and urine. These include neoepitopes generated by the collagenases (e.g., C2C, C12C and TIINE), fragments of the triple helix (Helix-II and Col 2-1), and C-terminal crosslinking telopeptides (CTX-II). See text for details.

identifying patients with rapidly progressive RA (13) and hip OA (14), possibly because these markers reflect different and partly independent mechanisms of cartilage erosion. Quite intriguingly, it has been shown that bone anti-catabolic agents, including various bisphosphonates, calcitonin, estrogens, and selective estrogen receptor modulators, when given to postmenopausal women with osteoporosis or patients with knee OA, produce a significant and dose dependent decrease of urinary CTX-II (for a review see refs. 15-17). Although some of these drugs are likely to have direct effects on chondrocyte metabolism, it may also be possible that the decrease in CTX-II reflects in part their activity in reducing the resorption of calcified cartilage.

The other major organic component of cartilage is aggrecan, which forms large link protein–stabilized aggregates with hyaluronan that are contained within the three-dimensional structure of CII. Aggrecan is highly hydrated because of its negatively

charged long polysaccharide chains, thus providing cartilage with the ability to resist compressive loads. The amino terminal end of the aggrecan monomer core protein is composed of two globular domains, G1 and G2, separated by an interglobular (IGD) domain (18). G2 is followed by a long central glycosaminoglycan attachment portion and by a COOH-terminal globular domain, G3. One of the earliest events in cartilage degradation associated with arthritis is the loss of aggrecan molecules, which is believed to result from proteolytic cleavages within the IGD domain. Two major cleavage sites have been identified: one between Asn^{341} and Phe^{342} attributed to MMPs, and the other between Glu^{373} and Ala^{374} attributed to aggrecanases, including a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and -5 (19). Different antibodies recognizing the N- and C-terminal aggrecan neoepitopes generated by these proteolytic cleavages have been developed and recently used in ELISAs. Pratta *et al*. (20) developed a sandwich ELISA that quantifies aggrecan fragments consisting of the C-terminal aggrecanase-neoepitope ^{374}ARGSVIL and a keratan sulfate (KS) region. Using an *ex vivo* model of human cartilage explants, they showed that these fragments were released after stimulation with interleukin-1, a cytokine believed to induce aggrecan degradation through aggrecanase-dependent pathways. They also detected the presence of this aggrecan neoepitope in human synovial fluids from patients with OA, but no data in serum were generated. Zeng *et al*. (21) generated a monoclonal antibody named AGG-C1 that recognizes specifically the aggrecanase-generated N-terminal neoepitope $TEGE^{373}$, which was used in a competitive ELISA. In a rabbit anterior cruciate ligament transection OA model, this epitope was detectable in synovial fluid and serum, but clinical data are still lacking. Finally, Sumer *et al*. (22) reported the development of two sandwich ELISAs. The first one (G1/G2) used a monoclonal antibody (F-78) detecting a common repetitive epitope in the G1 and G2 domains of aggrecan. This assay is believed to reflect either the synthesis of new aggrecan molecules and/or large degradation fragments. The second sandwich ELISA used the same format as the G1/G2 assay, except that it employed a different capture antibody (AF-28) that recognizes the MMP cleavage neoepitope ^{342}FFGVG. In a small cross-sectional study, levels of G1/G2 were reduced in patients with RA, suggesting decreased aggrecan synthesis, whereas values of the ^{342}FFGVG epitope were slightly but not significantly increased. No data in OA patients have yet been reported. In summary, current markers of aggrecan turnover have mostly been used in *ex-vivo* cartilage explants or in the synovial fluid of animals or patients with arthritis. Conversely, clinical data with serum measurements are still very limited, probably because it remains to be determined which circulating aggrecan fragment(s) is the most abundant and of most biological relevance.

Posttranslational Modifications of Proteins to Improve Tissue/Process Specificity of Biochemical Markers

Although CII and aggrecan are almost cartilage-specific, it is more challenging to develop specific biochemical markers of synovial tissue, as its extracellular matrix is composed mainly of type I and type III collagens, which are widely distributed (23). Developing sensitive biochemical markers of synovial tissue metabolism is very relevant in OA, as it has been shown that patients presenting with synovitis are more likely to progress (24). One of the posttranslational modifications of collagens is the glycosylation of hydroxylysine residues, including those forming pyridinoline (PYD) crosslinks. The analysis of the profile of PYD glycosylation in different human tissues by HPLC after alkaline hydrolysis revealed that PYD of synovial tissue is exclusively glycosylated by the disaccharide glucose-galactose (Glc-Gal), whereas PYD in bone is mainly comprised of the monosaccharide derivative Gal-PYD. This specificity of Glc-Gal-PYD was also demonstrated in *ex vivo* models of human joint tissue degradation. Urinary Glc-Gal-PYD has been found to be significantly elevated in patients with knee OA (24), especially in those presenting with knee swelling (25). Increased levels were also associated with decreased joint space width (26-28) and worse clinical symptoms (27).

Posttranslational modifications of proteins can be used not only to improve tissue specificity, but also to investigate pathophysiological processes, including oxidation. Chondrocytes and synoviocytes in OA can express high levels of inducible and neuronal forms of nitric oxide (NO) synthetase, which generates NO. NO can then react with superoxide radicals to form peroxynitrite, a potent oxidizing radical that can in turn react with tyrosine residues to form nitrotyrosine. Increased oxidative species have been found in the joint cavity of patients with OA and RA. Thus, measuring nitrosylated fragments of proteins may be useful in assessing oxidation-induced joint damage. This strategy has been used recently to generate NO-derived biochemical markers of type II (for cartilage) and type III (for synovial tissue) collagen degradation. Henrotin *et al.* developed two antibodies recognizing a sequence, either un-nitrosylated (called Coll 2-1) or nitrosylated (Coll 2-1 NO_2), of the triple helix of CII (29). Increased serum levels of Coll 2-1 and Coll 2-1 NO_2 have been reported in patients with knee OA and RA. Changes after 1 year in the urinary levels of these two products were related to rapid disease progression over 3 years (30). More recently, our group developed an antibody recognizing a sequence (named IIINys) of the type III collagen N-telopeptide containing two nitrosylated tyrosines (31). Immunohistochemistry demonstrated that IIINys is markedly expressed in the synovial membrane of patients with knee OA but not in muscle, which is a large source of type III collagen. We also found that IIINys peptides were abundant in the synovial fluid of patients with knee OA and in the serum of patients with RA.

Factors Influencing Systemic Levels of Biochemical Markers of Osteoarthritis

It must be highlighted that biomarkers, when measured in blood or urine since measurement of synovial fluid levels is often impractical, provide information on systemic skeletal tissue turnover and not necessarily on alterations in the signal symptomatic joint. Degenerative disease of the knees, hips, hands, and lumbar disks contributes independently and additively to urinary CTX-II levels, thus illustrating the total body contribution to systemic levels (32;33). The contribution of intervertebral discs is of particular relevance because disc degeneration is common with aging. The clearance of biomarkers from the joint compartment to the bloodstream is variable and can increase with inflammation or after joint mobilization. Most markers are metabolized in the liver or kidney before reaching a steady-state concentration in body fluids, which might also vary from one individual to another. All of these factors contribute to the variability of biochemical marker levels and must be considered when interpreting clinical data (Table 2). Disease progression in OA is not linear, but is characterized by cyclic phases of rapid and slower rates of progression. The maintenance of cartilage integrity is dependent on a tight balance between synthesis and degradation of the matrix molecules, which is altered in OA. In the early stages of disease, the anabolic activity of the chondrocytes is increased in an attempt to compensate for the degradative processes, but this capacity decreases as the disease advances. Thus, depending on the stage of the disease, markers of cartilage might respond differently.

Clinical Use of Biochemical Markers in Osteoarthritis

Based on the BIPED classification, most current markers of bone, cartilage, and synovial tissue can be referred to as *Investigative* as they are particularly useful in dissecting out the molecular processes involved in OA. What is the evidence that biochemical markers might be an important tool for the management of OA? Several cross-sectional studies have shown altered levels of biochemical markers in patients with OA compared to apparently healthy individuals. There is a large overlap, however, in marker levels between patients with an established disease, as documented by the classical radiological Kellgreen and Lawrence definition, and healthy controls (27). Measurements of any single available marker are therefore not sensitive enough for an efficient diagnosis of OA. Markers may prove to be more useful in identifying patients with early alterations of cartilage

tissue, potentially before damage is detectable by radiographs. In a cross-sectional population-based study of 201 subjects (34), it was shown that urinary excretion of collagenase C2C neoepitope was already significantly increased in patients who had MRI cartilage defects but no radiographic damage. We also showed that patients presenting with subchondral bone marrow abnormalities – a feature of early OA that has been shown to predict subsequent cartilage loss – are characterized by increased urinary CTX-II levels (35).

Recent longitudinal studies suggest that some new markers might have a role in the prognosis of OA. Progression in OA shows considerable variation across individuals, and the predictive capacity of clinical indices is poor. Obtaining biochemical markers that could identify rapid progressors would be particularly useful for the selection of patients to be included in clinical trials of disease-modifying therapy. Several retrospective analyses of prospective epidemiological or clinical trials have shown that subjects with CTX-II levels above the upper limit of controls had a 2–3-fold increased risk of progression at the knee or the hip, as assessed by radiography or arthroscopy after 1 to 6 years (6;7;36;37). A predictive value of serum PIIANP, COMP, hyaluronic acid, and MMP-3 was also reported in longitudinal studies. Despite careful positioning, the validity of radiography to assess cartilage loss remains questionable, and MRI, which provides higher resolution of joint damage, might be more relevant for future studies of biochemical markers. A recent study of 62 patients with knee OA reported that changes of CTX-II at 3 months were modestly associated with 12-month changes in medial cartilage thickness, although baseline values were not predictive (38). Because of the complex involvement of bone, cartilage, and synovial tissue in OA joint damage, it is likely that only a combination of several markers will adequately predict disease progression. Combining two markers of CII degradation, Helix-II and CTX-II, which reflect different pathways of cartilage degradation, was more effective than using one marker alone to predict disease progression in hip OA (14). In patients with knee OA, we found that the combination of a marker of CII degradation (CTX-II) with a marker of CII synthesis (PIIANP) was also more efficient than one marker alone for identifying patients with knee OA whose joint will deteriorate (6;7). In hip OA, the one-third of patients with the highest levels of CTX-II or hyaluronic acid had a risk of progression 1.8–2-fold over controls compared with the rest of the patients; this risk was 3.7-fold over controls in the 13% of the patients in whom both markers were elevated (37). An optimal combination of biochemical markers, together with clinical risk factors, imaging, and genetic markers, is likely to provide the clinician with a useful tool set for identifying OA patients at increased risk of disease progression. This combined strategy, which has been particularly powerful in predicting osteoporotic fractures, may vary according to the type of OA and the stage of the disease. In the Genetics osteoARthritis and Progression (GARP) study, the association of a panel of eight biochemical markers of bone, cartilage, and synovial activity with radiological OA at different joints was investigated (39). Using principal component analyses, it was found that the clusters of markers associated with knee and hip OA were different from those related to hand and spine OA.

One of the main issues that currently impairs efficient development of disease-modifying OA-modifying drugs (DMOADs) is the low sensitivity of plain radiography, requiring long-term studies to show a significant difference between placebo and active-drug-treated patients. Biological markers may prove capable of providing earlier information compared to a demonstration of slowing joint space narrowing by X-ray. The paucity of data on this topic is chiefly ascribed to the absence of medications with established structure modifying activity. While we are waiting for effective DMOADs, RA may serve as a model because effective drugs are available. In a randomized study of patients with early RA who received combined sulphasalazine-methotrexate-prednisone therapy, we found that the magnitude of the CTX-II decrease after 3 months was predictive of the changes in radiological scores after 5 years (40). Thus,

effective disease-modifying drugs can induce rapid changes in cartilage markers that are related to a valid clinical outcome. In two large randomized phase III trials of the bisphosphonate risedronate in patients with knee OA, a dose-dependent decrease of urinary CTX-II was also reported. Although risedronate did not demonstrate a significant reduction of radiological progression compared to placebo, there was a significant relationship between the level of CTX-II measured before and 6 months after treatment and joint space narrowing at 2 years (41).

Biochemical markers of OA are increasingly tissue-specific and the influence of the various factors that could obscure their clinical interpretation are better characterized. The panel of new markers is likely to expand with the optimization of genomic/proteomic based technologies. An optimal combination of biochemical markers, together with clinical, imaging, and genetic parameters, is likely to be useful for identifying OA patients at increased risk for disease progression and to speed the development of DMOADs.

Conflict of Interest: The author reports that he is an employee of Synarc.

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