Antibodies and immunoassays to monitor and measure types I and II collagen degradation and type II collagen and aggrecan synthesis

Examples of the use of the C1,2C, C2C, C2C-HUSA, CPII and CS846 molecular probes and biomarkers to study cartilage and soft tissue matrix turnover *in situ, in vitro* and *in vivo*

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Robin Poole, PhD, DSc

Professor Emeritus, McGill University, Montreal, Quebec, Canada

Consultant to IBEX Technologies Inc.

a.poole@mcgill.ca

Abbreviations: OA, osteoarthritis; RA, rheumatoid arthritis; SF, synovial fluid; NIH, National Institutes of Health; OARSI, Osteoarthritis Research Society International

1. Introduction

Starting in the early 1980s, antibodies and immunoassays thereof were created to establish ways of analyzing extracellular molecular turnover in hyaline cartilage. The resultant analyses resulted in an improved understanding of the structure and turnover of type II collagen, type I collagen, proteoglycans and associated molecules within the extracellular matrix of hyaline cartilages and other tissues. They have been especially valuable in studying growth plate development and articular cartilages in health and arthritis. Later, with the realization that they could be used to assay body fluids to gain new insights into cartilage turnover *in vivo*, the assays were used, more and more, as molecular biomarkers to study the turnover of these tissues in animals and people in health and disease.

We focused our attention on type II collagen and aggrecan as they are the predominant (by mass) structural components of the extracellular matrix of hyaline cartilages. Type II collagen provides cartilage with its tensile strength. Unlike with aggrecan degradation, excessive collagen damage is generally an irreversible event in the adult. So it was important to be able to detect and monitor the degradation of type II and to follow its synthesis as part of what is really an attempt at repair.

In contrast, the proteoglycan aggrecan endows cartilage with its ability to retain water and, with type II collagen, provides stiffness resisting the compression of articulation. We were fortunate to be able to discover a biomarker, now called CS846,

on newly synthesized aggrecan molecules that is lost as these molecules are degraded in the extracellular matrix.

Together these matrix molecules are largely responsible for the unique properties of hyaline cartilages. Both are actively synthesized and degraded even in health. Types I and II collagens are mainly degraded by collagenases whilst aggrecan is susceptible to cleavage of its core protein by other proteinases, such as MMPs and ADAMs, as part of physiology. The balance between anabolism and catabolism, is disturbed in pathology and this is reflected in the biomarkers of these molecules and the altered molecular events that accompany pathology.

As interest in these antibodies and biomarker assays grew they were licensed by Shriners Hospitals for Children for commercial development. As a result, existing assays and new assays were further developed and commercialized by IBEX. This review provides examples of the widespread use of these antibodies and biomarker assays by many researchers. It also traces their current independent appraisal by a public/private partnership involving the NIAMS/NIH, the Foundation for NIH and OARSI for use in clinical trials for the treatment of OA.

2. The antibodies and biomarker assays with general comments on their usage in tissue and body fluid analyses

2.1. Degradation of types I and II collagens: antibodies to collagenase-generated cleavage neoepitopes and the immunoassays C1,2C, C2C and C2C-HUSA

C1,2C assay: The initial cleavage of type I and II collagens by collagenases, such as MMP1, MMP8 and MMP13, generates a primary cleavage site containing a carboxy (C)- terminal neoepitope on the so-called ¾ piece. The assay for this neoepitope C1,2C was previously called Col2-3/4 C_{short} . The neoepitope is recognized by rabbit antibodies and is common to both types I and II collagens (Billinghurst et al, 1997). Since hyaline cartilage contains essentially no type collagen, the C1,2C assay can be used to analyze cleavage of type II collagen in cartilage samples (Billinghurst et al, 1997,; Mwale et al, 2002) and its degradation in culture (Dahlberg et al, 2000). In body fluids this assay would detect both types I and II collagen fragments containing this neoepitope. If a tissue contains no type II collagen the C1,2C antibody can be used to specifically detect cleavage of type I collagen by collagenases (Sorsa et al, 2003).

C2C assay : For use as a specific biomarker assay of body fluids a murine monoclonal antibody (Poole et al, 2004) was subsequently created that is specific for the same C-terminal cleavage product of type II collagen generated by collagenases. The C2C assay was initially called the Col2-3/4C $_{long\ mono}$ assay.

The C1,2C, C2C and other IBEX assays can be used for quantitative tissue analyses (Billinghurst et al, 1997; Squires et al, 2003; Antoniou et al, 1996; Aurich et al, 2005;

Dejica et al, 2008).Their use as biomarkers of *in vivo* metabolism includes assays of body fluids such as synovial fluid (SF), serum, plasma and urine, as detailed below, including the analysis of body fluids bathing hyaline cartilages such bronchiolar lavage fluid (Armstrong et al, 1999).

C2C-HUSA assay : Using the Mass-Spec information on type II collagen peptides present in urines of OA patients generated at IBEX and published by others (Nemirovskiy et al, 2007), a new sandwich assay was developed to specifically measure in urine the OA pathology-related 45 mer peptide containing the C2C neoepitope (Poole et al, 2016). Initial studies with this assay reveal that it detects the generation of a pathology-related cartilage collagen peptide or peptides in urine although little or no reactivity is seen in serum.

This urinary peptide is progressively increased with onset and progression of cartilage degeneration (Nemiroskiy et al, 2007), as seen in a population-based cohort examined radiologically and by MRI (Poole et al, 2016). Importantly, within this cohort it can discriminate between the normal knee, pre-radiologic articular cartilage degeneration and radiologic OA in a manner superior to that achievable with the C2C competitive inhibition assay applied to the same urine and serum samples. Moreover, the C2C-HUSA assay is more predictive of the progression of cartilage degeneration compared to the C2C assay for urine and serum (Poole et al, 2016). Importantly there was no correlation between the serum C2C assay and the C2C-HUSA urine assay, revealing their distinctness. A previous study with the C2C assay also revealed a lack of correlation between serum and urine measurements of C2C in the same patient (Cibere et al, 2009).

2.2. Synthesis of type II collagen as a procollagen: antibodies to the C-propeptide and a biomarker assay for its detection

CPII assay : When type II collagen is synthesized it contains amino (N) and carboxy (C) propeptides that are transiently present on the newly secreted molecule, enabling their registration to form collagen fibrils. Antibodies to the C-propeptide of type II procollagen were prepared and incorporated in an assay with which to detect the C-propeptide (Nelson et al, 1998) previously known as chondrocalcin before its true identity was established (van der Rest et al, 1986). This assay is used for tissue analyses and the assay of SF, serum and plasma.

With onset of increased cleavage in articular cartilages of type II collagen, such as in early OA, there is an accompanying increased synthesis of this collagen by chondrocytes. But in OA this newly synthesized collagen is quickly cleaved (Dahlberg et al, 2000). Type II collagen cleavage and synthesis is normally well balanced in healthy cartilage. In OA there is an increased emphasis on degradation (C2C) over synthesis (CPII), depicted by an increase in the C2C : CPII or C1,2C : CPII ratios, a reflection of arthritic changes favouring cartilage degradation and increased loss of

this molecule (Cahue et al, 2007). By using this ratio one can often observe changes in type II collagen metabolism that are otherwise not detectable.

Biomarker studies have strongly suggested that degradation of the newly synthesized type II collagen is what is mainly detected by the serum C2C assay as demonstrated by the high correlation between serum CPII and serum C2C (Cibere et al, 2009). This supports the earlier observation of preferential degradation of newly synthesized type II collagen in OA cartilage (Dahlberg et al, 2000).

2.3. Synthesis of aggrecan : an IgM antibody to a chondroitin sulfate epitope on newly synthesized aggrecan and its assay

CS846 assay: Intact and newly synthesized aggrecan in healthy tissues contains an epitope recognized by an IgM antibody which is now known as CS846. It is present on the largest newly synthesized aggrecan molecules and absent from smaller degraded fragments that can still aggregate with hyaluronan (Rizkalla et al, 1992). It can be used for assaying SF and serum or plasma (see below) as well as hyaline cartilages (Squires et al, 2003).

Principal component analyses of serum biomarkers CS846 and CPII in patients with knee and hip OA have revealed that biomarkers such as CPII and CS846 can be assigned to a putative cluster of biomarkers of anabolism (Otterness et al, 2000; van Spil et al, 2012). CS846 can also discriminate OA patients from controls (Otterness et al, 2000).

2.4. Immunohistochemistry

The methodology for the immunolocalization of C1,2C (Wu et al, 2002; Stoop et al 2001) , C2C (Dejica et al, 2012) and CPII (Nelson et al, 1998) is described in the aforementioned references. Similar methodology is used to detect CS846 but without prior removal of chondroitin sulfate with chondroitinase or hyaluronidase.

3. Physiological and external influences on the analyses of molecular biomarkers in body fluids

When measuring biomarkers it is important to standardize conditions, such as sampling time, as these may vary and influence the level of a given biomarker rather than innate changes in tissue metabolism alone. For this reason there have been studies, albeit very limited, that have investigated possible influences on the levels of these IBEX and other biomarkers and their relationships to variables, including those of a biomechanical nature. Overall these studies suggest that serum and urine sampling should be standardized, wherever possible, in a given study, especially in clinical trials (Kraus et al, 2011).

3.1. Diurnal variations and physical activity : Studies of different biomarkers in patients with knee OA (Kong et al, 2006) revealed that serum CS846, C2C and C1,2C did not change after the physical activity of arising from bed, unlike serum hyaluronan (HA), COMP and CPII which increased, along with urine C2C which continued to rise until early evening. The increase associated with getting out of bed almost certainly relates to the "pumping" of biomarkers into the circulation that have accumulated overnight in the SF. There were no diurnal variations observed for serum CS846, C1,2C, and C2C unlike urine CTX-II. Since HA is concentrated in the intestinal lymphatics and is further released into serum by peristalsis following a meal (Engstrom-Laurent, 1989; Pharmacia, personal communication) sampling at least 2-3 hours after the midday meal (mid afternoon) is recommended if this biomarker for synovitis is included in a study.

It is of special interest that interrelationships have been identified between limb/joint function and some these biomarkers. For example, the external knee adduction moment impulse relates to the ratio of urine CTX-II levels and serum CPII, even when controlling for various related variables (Hunt et al, 2013). Moreover higher peak vertical ground reaction force (vGRF) is associated with reduced serum C2C: CPII ratios in patients after ACL reconstruction (Pietrosimone et al, 2016). As indicated above, this ratio change would reflect an increase in cartilage type II collagen synthesis (CPII) in relationship to degradation of this molecule (C2C). In contrast, a reduction in peak vGRF and limb symmetry indices is associated with a higher ratio of serum C2C to CPII after reconstruction following ACL injury although the change it was not significant when corrected for walking speed (Pietrosimone et al, 2017). These studies suggest that the amount of biomechanical loading in the ACL reconstructed limb, compared to the contralateral limb, six months after reconstruction can be reflected by these biomarkers. How well such observations may relate to longer term clinical outcomes remains to be seen. But a 30 minute walk on a treadmill for patients with early knee OA results in changes in serum C1,2C and CS846 within a 5 hr. follow-up period. Interestingly, these changes are predictive of degenerative changes recorded 5 years later (Chu et al, 2017).

Exercised horses display an increase in serum C1,2C, CS846 and CPII (Frisbie et al, 2008) . Yet in a human study serum C2C, CPII and C2C:CPII did not change significantly throughout a multistage ultramarathon (Mundermann et al, 2017).

3.2. Effects of the menopause: Studies of serum C2C have revealed no detectable changes following the menopause in contrast to increases in the bone biomarkers bone alkaline phosphatase and cross-linked type I collagen N-telopeptide. (Kojima et al, 2008).

3.3. Skeletal growth and development : Carey et al (1997) showed that serum CPII levels are high in children from 0-14 years with an additional elevation during the pubertal growth spurt (10-14 years). This was followed by a subsequent marked reduction after 14 years, reaching a low in 35-60 year olds. Levels were unaffected by gender but varied from child to child at any given age. The main contribution to

the circulating CPII is considered to result from the considerable turnover of type II collagen in the epiphyses, and especially the growth plates of growing bones where type II collagen is synthesized and degraded as part of the prelude to the formation of woven bone. It is particularly in the hypertrophic zone that type II collagen is degraded, as part of normal physiology (Mwale et al, 2002), by the collagenase MMP 13. One of the outcomes of that cleavage is the generation of high levels of collagenase cleavage fragments detected in urine by the C2C-HUSA assay (Boeth et al, 2017). Their study of adolescent and adult volleyball athletes revealed a clear reduction in serum CPII, C2C and urine C2C-HUSA in those with closed growth plates compared to open. In adults C2C-HUSA showed a very marked reduction from adolescent levels along with the urine biomarker CTX-II. These biomarkers have much potential for assisting the study of skeletal growth.

4. Biomarkers and their association with arthritis

4.1. Haemophilic arthropathy (HA): In patients with haemophilia both serum CS 846 and urine CTX-II increase by 5 days after joint bleeding (van Vulpen et al, 2015). Serum CS846, urine CTX-II and serum C1,2C correlate with joint damage and joint space narrowing (Jansen et al, 2009). A combination of cartilage biomarkers CS 846, urine CTX-II and serum COMP increased the degree of correlation with joint damage. Of a broad series of biomarkers examined only CS846 revealed a significant correlation with MRI score in patients but only in those receiving treatment with hyaluronan (Oldenburg et al, 2016).

4.2. Haemochromatosis: The effects of iron depletion by phlebotomy in patients with hereditary haemochromatosis revealed that serum levels of ColI2-1 (another type II collagen degradation biomarker) and CPII are increased revealing that iron levels influence type II collagen turnover (Richette et al, 2010). A degradation:synthesis ratio would have helped clarify the analyses.

5. Onset, progression and treatment of inflammatory arthritis

5.1. Rheumatoid arthritis: Rheumatoid arthritis (RA) ordinarily involves multiple joints and more pronounced disease activity, especially prior to use of disease modifying treatments of which many are now available. Consequently, changes in these biomarkers with alterations in disease activity are often more pronounced than what we observe in conditions such as OA and ankylosing spondylitis. This provides more discrimination of disease presence and activity.

In RA the CS846 epitope averages an 8.6 fold increase in SF over serum (Poole et al, 1994). With increased joint involvement, serum levels of CS846 are generally more elevated in early RA than in OA with increases seen only in those early RA patients with relatively slow joint destruction and presumably reduced inflammation

(Mansson et al, 1995). In contrast, increases in serum CPII over controls are observed in those with both slow and rapid cartilage destruction. The differences in serum levels almost certainly reflect the influence of varying amounts of inflammation and are likely caused by proinflammatory cytokines such as interleukin 1 that, in low concentrations can inhibit matrix synthesis of aggrecan (Tyler et al, 1985) reducing levels of CS846 in those with early rapid progressive RA .
In SFs of patients with inflammatory arthritis a reduction of CPII was noted in early RA (Fraser et al, 2003). SF C2C and CS846 concentrations were similar in all groups analyzed. Only C2C levels of patients correlated with HAQ score, C-reactive protein levels, plasma viscosity (PV), synovitis scores and SF TNFα and MMP-1 levels. (Fraser et al, 2003). The direct correlation between the increases in TNFα and MMP-1 production and collagen degradation reflected by C2C suggests that collagenase cleavage of cartilage collagen is related to the activities of TNFα and MMP-1. The reduction in CII synthesis in early RA may contribute to the developing pathology, since a lack of synthesis of this molecule would inhibit maintenance of cartilage matrix.

Sera from patients with RA reveal increases in C2C over controls (Verstappen et al, 2006). Moreover, compared to RA patients with slow radiographic changes, those with rapid radiographic progression over a 4 year period had persistently elevated levels in sera of C2C, C1,2C and CS846, but not CPII. The values after one year predicted subsequent progression, especially in the case of C2C.

Support for the prognostic value of these biomarkers has also come from a biologic treatment study of patients with inflammatory arthritis. Examination of the balance between serum type II collagen (C2C) and type I collagen (C1,2C) degradation products and synthesis of type II collagen (CPII) revealed that after 1 month of treatment, changes in these three biomarkers predicted radiographic outcome in 88% of patients after 1 year (Mullan et al, 2007). An increase in C2C alone at 1 month predicted radiographic progression at 1 year. Clinical remission was predicted by a decline in serum C2C at 1 month.

Another RA treatment study by Niki et al (2012) showed that the serum ratio of C2C : CPII was decreased in early RA on treatment with infliximab, compared to baseline, regardless of the EULAR response grade . The ΔC2C : CPII over 54 weeks correlated with the changes in CRP, DAS28 levels, radiographic progression and patient function (HAQ). But C2C : CPII remained unchanged in established RA. These results suggest that the ability to control cartilage type II degradation(C2C) and promote its synthesis is most effective in early RA.

5.2. Psoriatic arthritis: This is a very neglected group when it comes to usage of these biomarkers. It has been shown that an increased ratio of serum CPII : C2C is indicative of those with erosive arthritis compared to psoriatics without joint disease (Chandran et al, 2010).

5.3. Ankylosing spondylitis: Baseline studies by Kim et al (2005) have reported elevations in serum CPII and CS846 and the CPII : C2C ratio compared to controls. But no changes were seen following treatment with infliximab. Others have found that patients treated with etanercept for 16 weeks revealed a reduction in serum C2C and an increase in serum CS846 (Maksymowych et al, 2005). A subsequent study over 2 years revealed that etanercept treatment caused a decrease in C2C after 12 months and after 24 months CPII was increased (Briot et al, 2008). Both studies point to a reduction in cartilage damage by etanercept (reduced C2C) and suggest onset of reparative responses reflected by increases in CS846 and CPII. More studies of these biomarkers as to their prognostic value and their use in clinical trials for AS are needed.

6. Onset and progression of human osteoarthritis and relationships to symptoms

OA is usually a much more silent and slowly progressive disease in terms of the rapidity of joint damage compared to erosive inflammatory joint disease. Often advanced joint damage may be detected only when symptoms present. Means of detecting early disease, such as MRI, are commonly restricted by accessibility and costs. Disease modifying responses to therapy may take up to two years to recognize. So large challenges exist when it comes to the management of OA.

The potential for biomarker usage in OA is therefore considerable and relates to whether they can be used to detect early onset, disease progression and responses to therapy as well as identifying sub-sets that may require specific management from a therapeutic standpoint. Much progress with biomarkers has been made in recent years. Many studies examined only single biomarkers with a lack of access of many basic scientists to clinical cohorts to compare most biomarkers in head to head studies. With the establishment of the OA Initiative in 2000, initially pioneered by NIAMS/NIH and now including partners from industry, Osteoarthritis Research Society International (OARSI) and the Foundation for NIH, many of these limitations have been removed. Significant progress has resulted in short lists of potential biomarkers for different indications with recommendations to the FDA (Kraus et al, 2011; 2016).

6.1. Knee OA onset : A common finding is that joint damage in knee OA is accompanied by increases in SF of biomarkers with even higher levels being seen in advanced OA. SF levels of CS846 reflect these changes (Lohmander et al, 1999) and in knee OA CS846 concentrations are on average 38 fold higher in OA than those observed in sera (Poole et al, 1994). This points to the damaged knee joint as the principal source of this biomarker.

Using MRI, positive correlations were observed between the C2C serum assay and cartilage degeneration in male OA knee T2 images (King et al, 2004). Increases in

serum C2C and C1,2C, but not CPII and CS846, are associated with radiographic knee OA (Kong et al, 2006) reflecting the increased cleavage of type II collagen by collagenases viewed *in situ* in diseased joints using the C1,2C and C2C assays and these antibodies used in immunohistochemical studies (Wu et al, 2002; Dejica et al, 2012). In a more recent study (Tamm et al, 2014), an elevation was reported for the urine C2C-HUSA assay in patients with early joint lesions of Outerbridge grade II or higher. These correlations were strongest when C2C-HUSA was expressed per creatinine.

As indicated above, principal component analyses of serum biomarkers CS846 and CPII in patients with knee and hip OA have revealed that CPII and CS846 can be assigned to a putative cluster of biomarkers of anabolism (Otterness et al, 2000; van Spil et al, 2012).CS846 can also discriminate OA patients from controls (Otterness et al, 2000).

6.2. Prediction of knee OA onset and progression :

MRI and radiographic analyses of a large population-based cohort with symptomatic knee pain were used to identify patients with and without pre-radiographic knee cartilage degeneration (pre-ROA) as well as those with radiographic knee OA (ROA). The risk of ROA versus no OA increased with increasing urine levels of C2C and C1,2C and was reduced in association with high levels of CPII (Cibere et al, 2009). The risk of pre-ROA versus no OA increased with increasing urine levels of C2C and C1,2C. However, the ratios of urine C2C or C1,2C : serum CPII were again more effective than individual biomarkers at differentiating the subgroups. In contrast, serum analyses of these degradation biomarkers failed to distinguish between the different groups. Also, there were no correlations between serum and urine measurements for each of the C2C and C1,2C assays. This pointed to the fact that the cleavage neoepitope-containing fragments were different in serum and urine.

Others have also found that early radiographic onset of knee OA is accompanied by increases in serum C2C and C2C:CPII and a decrease in CPII (Ishijima et al, 2011). In those patients with no evidence of radiographic OA, but experiencing knee pain, C2C and CPII were increased pointing to early changes in cartilage turnover that are characteristic of onset of articular cartilage degeneration in one or more joints. Urine analyses of C2C by He et al (2014) also revealed that they were higher in knee OA than in controls.

The investigation into whether these biomarkers could be used to predict knee OA progression was encouraged by the observation that the ratios of C2C: CPII and C1,2C: CPII both showed an almost significant relationship to radiographic assessment of disease progression not seen with the individual biomarkers (Cahue et al, 2007).

With the more recent creation of the C2C-HUSA urine assay it was discovered that baseline values are associated with the progression of cartilage degeneration in knee OA over 3 subsequent years: baseline C2C-HUSA levels were also higher in

progressors versus non-progressors (Poole et al, 2016). Furthermore, in an OA initiative head to head assessment of 18 biomarkers, C2C-HUSA was one of only 2 biomarkers that predicted case status, the other being CTX-II, and also individual group status, including pain worsening, joint space loss and their combination (Kraus et al, 2016). Tamm et al (2014) also observed positive correlations with symptoms as well as joint function. These correlations were strongest when C2C-HUSA was expressed per creatinine.

Following earlier studies of serum COMP, there is recent evidence that mechanically-induced changes in serum cartilage matrix biomarkers can predict regional changes in cartilage thickness 5 years later in human subjects with early knee OA (Chu et al, 2017). Subjects were exercised on a treadmill for 30 minutes and blood samples were obtained 30 minutes and 5.5 hours after exercise. MRIs of the index knees were acquired at baseline and after 5-years. Serum biomarker concentrations of C1,2C and CS846 were measured. Changes in biomarker concentrations (0.5h vs 5.5h) were determined and correlations between changes in cartilage thickness and biomarker levels (as a percentage of 0.5 h post-activity levels) were assessed. For knees where the catabolic and anabolic marker concentrations increased, specific regions of articular cartilage were thinner. The study supports the hypothesis that a mechanical stimulus can produce a change in both markers of degeneration and synthesis that correlate with subsequent changes in cartilage thickness. Many of the thickness changes were in the lateral compartment of both the tibia and femur. Given that the patients in this study were selected with mild to moderate medial compartment OA, it appears that in the relative early stages of the disease (average KL 2.5), these biomarkers reflect both thickening and thinning in both compartments. Assuming that both cartilage thickening (likely due to damaged cartilage's propensity to initially swell) and thinning can reflect degenerative changes, the data supports further exploration into the use of a mechanical stimulus to elicit biomarker changes potentially useful for predicting OA progression.

6.3. Knee and hip OA haplotypes : When knee or hip OA patients were haplotyped for mitochondrial haplogroups , the C2C, CPII and the C2C : CPII ratio were significantly increased in sera of OA patients carrying the haplogroup H compared to OA carriers of the J haplogroup (Fernandez-Moreno et al, 2012). The collagenase MMP-13 is also more elevated in patients of the more common haplogroup H who are more likely to need total joint replacement than non-H haplotypes (Soto-Hermida et al, 2015). These observations fit well with the known close association of collagenase activity, especially MMP-13, with cartilage degeneration (Mitchell et al, 1996; Dahlberg et al, 2000).

Patients with hip OA also exhibit differences in serum levels of C2C and CPII according to whether it is bilateral or unilateral hip OA or multiple site OA (Conrozier et al, 2008). The unilateral hip OA group exhibited a significant inverse correlation between minimum joint space width and serum C2C. It remains to be established whether different haplotypes influence the creation of these sub-groups.

6.4. Hand OA : A review of serological biomarkers of erosive and non-erosive hand osteoarthritis by Ramonda et al (2013) concluded that C1,2C was one of the most discriminatory biomarkers for the study of hand OA.

6.5. Spine OA : Studies of intervertebral disc space narrowing in patients with OA of the lumbar spine revealed significant differences in serum CPII and C2C between different levels of severity (Goode et al, 2012).

7. Post traumatic knee OA

Patients at risk for OA following knee ACL injury, with or without abnormal joint space width (JSW) reflective of cartilage loss showed an increased ratio of urine C1,2C : serum CPII compared to controls after 1 and 4 years (Tourville et al, 2013). Those with abnormal JSW had increased urine CTX-II : serum CPII compared to controls. Urine CTX-II may more likely reflect early osteophyte formation in view of the principal origin of this biomarker in calcified cartilages (Bay-Jensen at al, 2008) and its demonstrated association with biomarkers of the turnover of calcified tissues (van Spil et al, 2012).

In another study following knee ACL rupture, in which baseline serum pre-injury samples were available, subsequent serum changes in C2C, C1,2C, CPII and CS846 were observed compared to age-related changes in uninjured controls (Svoboda et al, 2013). There were significant decreases in C1,2C and C2C over time in the ACL-injured group compared to the controls. CS846 from baseline to follow-up was also significantly different between the ACL-injured patients and uninjured controls as was the change between groups in the ratio of C2C : CPII over time. No preinjury differences in the ratio of C1,2C : CPII or C2C : CPII were observed between groups although postinjury differences were observed for both ratios.

Increases in knee SF C2C levels occur within a day following injury and persist up to 7 years (Kumahashi et al, 2015). Interestingly, C2C concentrations in SF and serum were correlated. These changes reflect increased collagenase activity, detected by C1,2C analyses of articular cartilage following ACL rupture (Nelson et al, 2006).

Arthroscopic analyses of pre-radiographic knee cartilage degeneration following ACL injury have revealed significant associations of increased synovial fluid C2C with the presence of three or more high Outerbridge graded cartilage lesions (Yoshida et al, 2013).

A radiographic study of patients who had a reconstructed ACL following injury revealed 2 groups: one with normal joint space width (JSW) and one that was abnormal (Tourville et al, 2013). In comparison to matched controls, both groups had an increased ratio of urine C1,2C : serum CPII relative to the controls at 1 and 4 year follow-ups. Another study by this group of post ACL damage has provided strong evidence in support of an association between the SF markers of type II collagen

metabolism (C1,2C and CPII) and the KOOS subscale of pain between the OA low and high risk groups (Wasilko et al, 2016).

Patients with a reconstructed ACL (Pietrosimone et al, 2016a) have lower serum C2C : CPII ratios that are associated with higher peak vertical ground-reaction force (vGRF) in the injured limb. Reduced vGRF limb symmetry indices were associated with higher C2C : CPII ratios after ACL injury but this was not significant after accounting for walking speed (Pietrusimone et al, 2017). These observations suggest that the higher peak loading accompanies reduced type II collagen breakdown relative to synthesis. Whether vGRF is also related to fewer symptoms remains to be established. Pietrosimone et al (2016b) also showed that serum C2C is elevated in those reconstructed ACL patients with a slower walking speed suggesting that there is increased damage to articular cartilages of these individuals.

8. Treatment of osteoarthritis

The use of biomarkers in the development of treatments for arthritis has attracted much attention. Recommendations on the use of molecular biomarkers in the development of drugs intended for the treatment of OA were submitted to the FDA 6 years ago (Kraus et al, 2011). They include the IBEX biomarkers C2C, C1,2C, CPII and CS846. These, and many other recommended biomarkers, with the addition of more recent assays such as the C2C-HUSA urine assay, were the subject of further study investigating the predictive ability of biomarkers as part of the NIH public/private OA initiative involving OARSI, the Foundation for NIH and industry. Out of 18 assays examined the C2C-HUSA assay was one five identified as of importance in future studies: there was a second cartilage assay, urine CTX-II, and the urine CTX-I and NTX-I assays for bone resorption and serum hyaluronan for synovitis (Kraus et al, 2016).

There have been very few published treatment studies involving IBEX biomarkers. Most have been investigative. In an early study patients with knee OA receiving oral salmon calcitonin displayed a significant reduction in urine C2C, not seen in the placebo group. This was accompanied by significant improvements in joint function and reduced levels of the collagenase MMP-13 (Manicourt et al, 2006).

Glucosamine has been the subject of much debate as to its efficacy in treating OA. An initial study of glucosamine sulfate administered to those with knee OA up to 24 weeks revealed no convincing evidence of an effect on serum or urine C1,2C or C2C levels or ratios thereof (Cibere et al, 2005). A study of N-acetylglucosamine administered over 16 weeks to persons without symptomatic or radiographic evidence of OA also revealed no effect on serum C2C and CPII (Kubomura et al, 2017). But subgroup analyses revealed that in those people with elevated C2C and reduced CPII, C2C levels were significantly decreased at 8 and 16 weeks compared to the placebo group. Therefore there is evidence to indicate a suppression of type II collagen degradation in this sub-group. The identification of sub-groups with active

disease will be of great importance in future clinical treatment trials. By using biomarkers such as C2C-HUSA to recruit patients with active disease should avoid most clinical trials where as few a 15-25% of patients exhibited progression over a 2-3 year period.

A recent randomized double-blind placebo-controlled clinical trial evaluated the chondroprotective action of salmon nasal cartilage proteoglycan in individuals with knee joint discomfort but without diagnosis of knee osteoarthritis (Tomonoga et al, 2017). C1,2C levels dropped significantly in the treatment group compared with the placebo group following a 16 week intervention of subjects with high levels of knee pain and physical dysfunction and subjects with constant knee pain. The C1,2C : PIICP ratio decreased in the treatment group, whereas it increased a little in the placebo group following treatment.

More direct analyses of knee articular cartilage collected at arthroplasty following a short treatment period prior to surgery can provide an alternative approach to the use of these biomarkers. Such was the case with patients treated for three weeks prior to arthroplasty with a metalloproteinase inhibitor compared to a placebo. The findings revealed significant increases in cartilage CS846 although there was no evidence of any changes in collagen cleavage or synthesis, yet collagen content was increased (Leff et al, 2003). Short term studies of patients scheduled for arthroplastic surgery should be considered as part of preliminary studies of proof of principal of potential for therapeutic efficacy.

As described below, canine treatment studies of hip OA reveal that serum C2C correlates with clinical improvement (Vilar et al, 2016)

9. Prediction of susceptibility to joint injury

The use of these serum assays has unexpectedly revealed that patients at increased risk for ACL rupture can be identified prior to injury by differences in serum C2C, C1,2C, CPII and CS846 levels (Svoboda et al, 2016). This was made possible by the availability of serum samples taken from healthy military cadets prior to sustaining ACL injury. In another study of young athletes who suffered shoulder injury and instability, preinjury serum C2C levels were significantly lower than in the control group. CPII serum levels showed no differences in pre-injured patients (Owens et al, 2017).These novel and unexpected findings suggest that fundamental genetic and/or biomechanically related differences exist that influence cartilage metabolism. Identification of such differences offer an awareness of increased risk of such injuries. Such observations could have profound value in the reduction of sports injuries.

10. Animal studies of experimental and natural osteoarthritis, osteochondrosis and inflammatory arthritis

Although these assays were developed for human studies based upon human neoepitope sequences and proteins, the existence of identical or antibody cross-reactivity to almost homologous sequences in epitopes across species has enabled their use in a number of animal biomarker studies. Some of these are described here.

10.1. Equine

Osteochondrosis (OCD): Compared to healthy horses, SF from cases of equine OCD exhibit increases in CPII and decreases in CS846 pointing to an increase in type II collagen synthesis and impaired aggrecan synthesis (Laverty et al, 2000). Using the C1,2C assay, explant studies demonstrated increased degradation of type II collagen in OCD (Laverty et al, 2002). In foals with tarsocrural OCD the CPII : C2C ratio in SF tended to be higher in affected joints relative to controls at all ages and significant at 22 weeks of age: CS846 was reduced at 18 weeks (de Grauw et al, 2011). Donabedian et al (2008) found that body weight was correlated negatively with serum C2C and withers height was positively related to CPII : C2C ratios. In other studies SF C2C, but not CPII, was increased in OCD joints but not in fracture joints where CPII was elevated in fracture unlike OCD joints. In neither group was there a correlation between these biomarkers and arthroscopic findings (Lettry et al, 2010)

In foals serum C1,2C was indicative of OCD severity at 5 months of age (Billinghurst et al, 2004). In those with lesions at 11 months of age, severity correlated negatively with C1,2C and positively with CPII.

Intraarticular Steroids: The injection of methylprednisolone into healthy joints of adult animals causes an increased release of aggrecan degradation products detected in SF with the CS846 assay whereas CPII is reduced (Robion et al, 2001). This points to the potentially harmful effects of long term corticosteroid usage. Further work with intraarticular triamcinolone acetonide demonstrated significant increases in SF CS846, C1,2C and CPII concentrations. The uninjected contra-lateral joints also exhibited an increase in C1,2C and CPII demonstrating a systemic effect (Celeste et al, 2005).

Osteochondral injuries: Osteochondral fragmentation can be diagnosed in a majority of animals by serum elevations of CS846 and CPII (Frisbie et al, 1999). In animals with injuries in MCP and MTP joints, SF analyses with C2C revealed increases in injured joints that correlated with the severity of injury based on arthroscopic and radiographic scores (Trumble et al, 2009).

Osteoarthritis: In early experimental OA in horses, SF levels of C1,2C, CPII and CS846 were all increased (Frisbie et al, 2008).

10.2. Canine

The natural development of OA in dogs of medial coronoid disease in dogs with dysplastic elbows is accompanied by an increase in the concentration of SF C2C (Prink

et al, 2010). There is a moderate correlation between cartilage damage grade and increasing C2C concentations . Earlier work by this group failed to reveal any changes in C2C in serum, urine and joint fluid in dogs with cranial cruciate ligament rupture (Hayashi et al, 2009).

Induction of unilateral OA in dogs leads to elevations in SF C2C. It starts at 3 weeks followed by serum and urine increases in C2C at 12 weeks (Matyas et al, 2004). Serum CS846, not measured in SF, is also elevated at 3 and 12 weeks. C1,2C is also increased in SF experimental OA (Chu et al, 2002).

As reported above, serum C2C has proved of value in monitoring effectiveness of treatment in canine hip OA (Vilar et al, 2016) with decreases in C2C with clinical improvement measured by force plate analysis.

10.3. Murine

Murine OA also displays elevations of serum C2C (Ameye et al, 2007). Earlier immunohistochemical work with antibody C1,2C showed increased staining in degenerating cartilage in stifle joints of ageing mice (Stoop et al, 1999). Similar results were observed for cartilage degradation in antigen-induced inflammatory arthritis (van Meurs et al 1999).

10.4. Rat

In a rat model of intra-articular injection of streptococcal cell walls, an inflammatory arthritis is induced. Serum C2C is increased with onset of acute inflammation and more so in chronic disease. This increase was much reduced on treatment of the arthritis (Song et al, 1999). In exercised ACL transected rats the ratio of serum C2C : CPII increases with the development of experimental OA (Yamaguchi et al, 2013). Immunohistochemical analyses of experimental rat OA have demonstrated much increased staining of articular cartilage in stifle joints using the C1,2C antibody (Stoop et al, 2001).

10.5. Guinea pig

The naturally OA prone Hartley strain was compared to strain 13 OA-resistant animals. With the onset of OA lesions in stifle (knee) joints, serum levels of C2C and CPII changed with the result that the C2C : CPII ratio increased in Hartley animals compared to strain 13. (Huebner and Kraus, 2006). Induction of post-traumatic OA by anterior cruciate transection results in the elevation of SF C2C (Wei et al, 2010).

10.6. Porcine

MRI studies of healthy animals and those with naturally occurring OA (Hatcher et al, 2017) demonstrated that T1rho relaxation images of OA knee condylar cartilages exhibit an increase in relaxation times compared to normal joints. In these regions cartilage proteoglycan content and aggregate modulus decreased while percent degraded collagen and water content increased compared to healthy joints. These changes corresponded to proteoglycan decreases and C2C increases in SF compared to healthy joints.

10.7. Lapine

Rabbits with monoarticular inflammatory arthritis also show an increase in C2C in joint fluids of the affected stifle joint, but not in serum (Kojima et al, 2001), probably because the changes in the rabbit are limited and monoarticular.

10.8. Elephant

Serum C2C and CPII have been used in Asian elephants to study chronic lameness (Kilgallon et al, 2015). Both CPII and C2C were decreased in lame animals.

11. Concluding remarks

This updated and expanded overview of research involving these IBEX biomarker assays and antibodies reveals their widespread use and value, particularly as molecular biomarkers, particularly in improving our understanding of joint disease where they have been used most often. Their value in research on OA onset, progression and treatment has more recently become more apparent. Clinical trials for OA have been blighted by the lack of patients with active progressive disease, mainly because some OA, such as knee OA, is not always progressive. The potential offered by biomarkers such as C2C-HUSA, enables the identification of sub-groups with active disease. This will be of great importance in future clinical treatment trials. By using C2C-HUSA to pre-screen and recruit patients with active disease should avoid clinical trials where as few as only 15-25% of patients exhibit measurable progression over a 2-3 year period. (Poole et al, 2016)
As I have indicated, these biomarkers also have value in studying the pathology of tissues other than joints, such as lung and gingiva, wherever cartilage or type I collagen and their turnover are of interest.

I hope that these notes may be of use in helping you reach an improved understanding of cartilage and soft connective turnover in health and disease and in the development of disease modifying treatments of OA.

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