Use of IBEX biomarker assays and antibodies thereof to monitor growth, resorption, degradation and damage to connective tissues: a focus on cartilage degradation and arthritis

February, 2020

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; FNIH, Foundation for NIH; OARSI, Osteoarthritis Research Society International; ACL, anterior cruciate ligament

1. Introduction

Starting in the late 1970s and early 1980s, antibodies and immunoassays thereof were developed to detect and measure matrix molecules, their synthesis and degradation, by groups such as my own and that of Dick Heinegard in Lund, Sweden. We wanted to establish ways of analyzing extracellular molecular turnover in soft connective tissues and hyaline cartilages within joints and, in my case, the growth plate. The resultant analyses resulted in an improved understanding of the turnover of type II collagen, type I collagen, proteoglycans and associated molecules within these tissues. The assays and antibodies have been especially valuable in studying growth plate development and articular cartilages in health and arthritis. With our independent 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.

I focused my 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 type II collagen damage is generally an irreversible event in the adult. So, it was important to be able to detect and monitor the degradation of this collagen 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 and cathepsin K 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' synthesis and degradation and the altered molecular turnover that accompanies 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 current widespread use of these antibodies and biomarker assays in research. It also traces their 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 examples of 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 antibody and immunoassay: 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 antibody and 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 I 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 can 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 (Ma et al, 2003).

C2C antibody and immunoassay: 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 also be used for quantitative tissue analyses (Billinghurst et al, 1997; Squires et al, 2003; Antoniou et al, 1996; Aurich et al, 2005; Nelson et al, 2006; Dejica et al, 2008, 2012) whereby the neoepitopes can be extracted from tissues enzymatically or detected directly in culture media. Their use as biomarkers of *in vivo* metabolism includes assays of body fluids such as 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 urine immunoassay: 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 45mer peptide containing the C2C neoepitope (Poole et al, 2016). Studies with this sandwich 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. Comparative studies (Kraus et al, 2017a,b; Boeth et al, 2017) also reveal that it has significant similarities and important differences to the CTX-II urine immunoassay.

In development, urine levels of both C2C-HUSA and CTX-II are much higher than in adults, especially in those with open growth plates (Boeth et al, 2017). Peptides containing the C2C-HUSA epitopes are progressively increased in urine with onset and progression of cartilage degeneration (Nemiroskiy et al, 2007), as also seen in a population-based cohort examined radiologically and by MRI (Poole et al, 2016). Importantly, sub-populations of this cohort can be discriminated into those with normal knees, 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 applied to urine and serum (Poole et al, 2016). There is 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 antibodies and immunoassay: When type II collagen is synthesized it contains amino (N) and carboxy (C) propeptides that are transiently present on the newly secreted molecules, enabling their registration to form collagen fibrils. Rabbit 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 by sequencing (van der Rest et al, 1986). This assay is used for tissue analyses (Nelson et al, 1998; Squires et al, 2003) and the assay of SF, serum and plasma.

In young children and adolescents, serum CPII is elevated compared to adults (Carey et al, 1997; Boeth et al, 2017), but very much less than the elevation observed for C2C-HUSA in urine (Boeth et al, 2017). With onset of increased cleavage in articular cartilages of type II collagen in OA, there is an accompanying increased synthesis of this collagen by chondrocytes (Nelson et al, 1998). But in this case, this newly synthesized collagen is quickly cleaved, unlike in healthy cartilage (Dahlberg et al, 2000) where type II collagen cleavage and synthesis are normally well balanced. 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 serum 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 turnover that are otherwise not apparent.

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 antibody and immunoassay: 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). The location of this epitope in healthy cartilage adjacent to the C-terminal G3 domain was confirmed by analysis of this large IgM antibody bound to fetal aggrecan by rotary shadowing and subsequent electron microscopy (M.Morgelin, A.R.Poole, B.Mansson, D. Heinegard, unpublished). The assay can be used for SF, 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 revealed that these biomarkers 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).

Except for serum CPII, all of the above assays for serum and/or urine have been independently analyzed as part of the FNIH/OARSI initiative for reference intervals for OA-related molecular biomarkers (Kraus et al, 2017b).

2.4. Immunohistochemistry

Extensive studies have used the above antibodies to study the synthesis and degradation of aggrecan and type I and II collagens. Examples of the methodology for immunolocalization with the antibodies 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 to increase tissue permeability to the antibodies.

3. Physiological and external influences on 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 the result of innate changes in tissue metabolism alone. For this reason, studies, albeit limited, have investigated possible influences on the levels of 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 mechanically induced increased "pumping out of joints" of biomarkers into the circulation: biomarkers that have accumulated overnight in the SF. Diurnal variations have also been 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, likely from mesentery, by peristalsis following a meal (Engstrom-Laurent, 1989; Pharmacia, personal communication) sampling at least 2-3 hours after the midday meal (midafternoon) is recommended if this biomarker for synovitis is included in a study.

A recent study of hand OA revealed that CPII levels are significantly increased in serum, along with COMP and other biomarkers, after mechanical exercise of the OA hand (Bender et al, 2019) and can be used to distinguish Bouchard's nodal OA from premenopausal controls.

Other interrelationships have been identified between limb/joint function and biomarkers. For example, the external knee adduction moment impulse relates to the ratio of urine CTX-II levels to 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, 2016a). 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 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 is reflected by these biomarkers. How well such observations may relate to longer term clinical outcomes remains to be seen. But a 30 min. 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, 2018).

Exercised horses display an increase in serum and SF of C1,2C, CS846 and CPII (Frisbie et al, 2008). Yet in a human study serum C2C, CPII and C2C:CPII showed no significant changes throughout a multistage ultra-marathon (Mundermann et al, 2017).

3.2. Effects of gravity: Preliminary information suggests that long duration space flight influences cartilage metabolism (Niehoff et al, 2016). Pre and post flight serum samples from astronauts spending more than 5 months at the International Space Station revealed increases in C2C, CPII and COMP up to 30 days after return from spaceflight, reaching significance only for COMP. Whether individual increases (each individual having a built in control) were observed was not reported.

3.3. Effects of 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).

3.4. Effects of gender and race: No effects on IBEX biomarkers, including urine C2C-HUSA, were observed in a large FNIH/OARSI biomarker consortium study of reference levels (Kraus et al, 2017b). In contrast, other biomarkers such as urine CTX-II were shown to vary with race and gender. These differences help distinguish urine C2C-HUSA from urine CTX-II which often gives similar results. Clearly the lack of need to correct for gender and race is an advantage when using the IBEX assay.

3.5. Skeletal growth, development and ageing: Carey et al (1997) showed that serum CPII levels are elevated in children from 0-14 years with maximal 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. In development, 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 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 urines of adolescents 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 those still open revealed by MRI. In adults C2C-HUSA showed a very marked reduction from adolescent levels along with the urine biomarker CTX-II. IBEX biomarkers have much untapped potential in the study of skeletal growth.

In adult ageing, only serum C1,2C showed changes, namely a negative correlation (Kraus et al, 2017b): serum CS846, serum C2C, urine C2C, urine C2C–HUSA were unaltered. In this same study reference levels were established for the OA Initiative biomarkers study.

4. Biomarkers that reflect joint pathology

4.1. Haemophilic arthropathy (HA): In patients with haemophilia both serum CS 846 and urine CTX-II are increased 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). The combination of cartilage biomarkers CS 846, urine CTX-II and serum COMP increased the degree of correlation with radiographically defined joint damage. Of a broad series of biomarkers examined, only CS846 revealed a significant correlation with MRI scores in patients requiring treatment who had pronounced bleeding and joint pathology. (Oldenburg et al, 2016). These relationships may relate to induction of increased synovitis and subsequent cartilage damage as a result of persistent joint bleeds.

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

5. Biomarkers detect the onset, progression and treatment of inflammatory arthritis

5.1. Rheumatoid arthritis (RA): RA ordinarily involves multiple joints and more pronounced disease activity. With what is called the increased "joint load", 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, activity and response to treatment.

In RA the CS846 epitope shows a mean 8.6-fold increase in concentration in SF over serum pointing to its source (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 less inflammation (Mansson et al, 1995). In contrast, this study reported increased serum CPII over controls is observed in those with both slow and rapid cartilage destruction. The differences in serum levels of CS846 are likely caused by proinflammatory cytokines such as interleukin 1 (IL-1) that, in low concentrations can selectively inhibit synthesis of aggrecan (Tyler, 1985) reducing levels of CS846 in those with early rapid progressive RA.

In contrast to serum, SFs of patients with inflammatory arthritis exhibit a reduction of CPII 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 derived from inflamed synovium. The reduction in type II procollagen synthesis observed in joints in early RA, nor detected systemically, may well be caused by pro-inflammatory cytokines such as IL-1 (Tyler and Benton, 1988). This would contribute to the developing pathology, since a lack of synthesis of this molecule would inhibit maintenance of articular cartilage matrix.

Analyses of 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 3 biomarkers predicted radiographic outcome in 88% of patients after 1 year (Mullan et al, 2007). An increase in serum 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) demonstrated 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 the C2C:CPII ratio remained unchanged in established RA. These results suggest that the ability to control cartilage type II degradation (C2C) and promote its synthesis is more effective in early RA.

***5.2. Juvenile idiopathic arthritis (JIA)*:** Recent observations have indicated that JIA may be amenable to study using biomarkers although observations may be complicated by biomarkers generated as part of skeletal growth, especially from growth plates. Struglics et al (2019) reported that plasma levels of the ARGS-aggrecan neoepitope, C2C, COMP and TRAP5b were all increased in JIA compared to healthy children. Compared to knee-injured juveniles, SF C2C and TRAP5b were increased in JIA, while ARGS and COMP were decreased. In these JIA patients, local (SF) and systemic (plasma/urine) levels of C2C were not correlated.

***5.3. Psoriatic arthritis*:** An increased ratio of serum CPII:C2C is indicative of those with erosive arthritis compared to psoriatics without joint disease (Chandran et al, 2010).

5.4. Ankylosing spondylitis: Baseline studies by Kim et al (2005) reported elevations in serum CPII, CS846 and the CPII:C2C ratio compared to controls. But no changes were seen following treatment with infliximab. Others 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. Biomarkers to study the onset and progression of human osteoarthritis and relationships to symptoms

OA is usually a more silent and slowly progressive disease compared to the rapidity of joint damage often seen in erosive inflammatory joint disease. Often advanced joint damage requiring arthroplasty may be detected only when symptoms present. Means of detecting early disease, such as MRI, are often restricted by accessibility and costs. Disease modifying responses to therapy may take up to two years to recognize. As OA is a phasic disease it is essential to conduct clinical trials with patients showing disease progression. It is likely that clinical trials have failed to show disease modification since the majority of patients in most trials have shown no progression in the placebo group. So large challenges exist when it comes to the conduct and expense of clinical trials and the management of OA once disease-modifying drugs are available. Hence the great importance of the OA Initiative.

The potential for biomarker usage in OA is therefore considerable and relates to whether biomarkers, and specifically in this case, whether molecular biomarkers can be used to detect early onset, disease progression and reasonably rapid measures of responses to therapy. Moreover, because of the heterogeneity of OA (common outcome/phenotype resulting from variable molecular pathology from different genotypes) it is important to identify sub-sets. These may require specific management from a therapeutic standpoint.

Much recent progress has been made with the development of new molecular biomarkers and how they are used. Many earlier studies examined only single biomarkers with a lack of access for many basic scientists to clinical cohorts to compare 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, OARSI and the Foundation for NIH (FNIH), 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; 2017a). Unfortunately, recent financial limitations appear to have stalled additional analyses of molecular biomarkers that are required to complete FDA submissions. Moreover, important biomarkers offering additional clinical insights into symptoms have been omitted in recent predictive combinatorial models, presumably in the desire to limit the combination of biomarkers required to identify disease progression and outcomes (Kraus et al, 2017a). As our patients always tell us, it is the pain and fatigue suffered by those with arthritis that is their top priority. This must also not be ignored.

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 SF concentrations are on average 38-fold higher in OA than those observed in sera (Poole et al, 1994). This points to the diseased knee joint as the principal source of this biomarker as in RA described above.

Using MRI, positive correlations were observed between serum C2C 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 urine 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

Onset: 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 indicative of repair (Cibere et al, 2009). The risk of MRI defined pre-ROA versus no OA increased with increasing urine levels of C2C and C1,2C. However, the ratios of urine C2C or C1,2C and serum CPII were again more effective than individual biomarkers at differentiating the subgroups. In contrast, serum analyses of these collagen 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 detected by the immunoassays were probably different in serum and urine.

Others have also found that early radiographic onset of knee OA is accompanied by increases in serum C2C and the ratio of C2C:CPII and a decrease in CPII (Ishijima et al, 2011). In those patients with no evidence of radiographic OA, but experiencing knee pain, serum 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. He et al (2014) also found that urine C2C is higher in knee OA than in controls.

Progression: 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: this was not seen with the individual biomarkers (Cahue et al, 2007). In a study of normal reference levels for potential OA biomarkers as part of the FNIH/OARSI/industry knee OA initiative (Kraus et al, 2017b), the mean concentration of urine C1,2C varied in a manner consistent with the possibility of the presence of early but radiographically negative OA in some participants in a subgroup without follow-up time points. This suggests that this would be worth investigating further as a potential biomarker for predicting incident radiographic OA that would involve changes in turnover of soft tissue type I collagen (found in synovia, menisci, ligaments, tendons, subchondral bone) as well as type II cartilage collagen.

With the more recent creation of the C2C-HUSA urine assay it was discovered that baseline values are predictive of the progression of cartilage degeneration in knee OA over 3 subsequent years: baseline C2C-HUSA urine levels per creatinine were also higher in progressors versus non-progressors (Poole et al, 2016).

The FNIH/OARSI/industry consortium OA initiative, conducted an independent blinded assessment of 18 potential OA cartilage, bone and other biomarkers at baseline, 12 months (M), 24M and 48M. Primary analyses used multivariable regression models to evaluate the association between biomarkers (baseline and time integrated concentrations (TICs) over 12 and 24 M, transposed to z values) and case status, adjusted for age, sex, body mass index, race, baseline radiographic joint space

width, Kellgren-Lawrence grade, pain and pain medication use. The study showed that only C2C-HUSA and CTX-II individually predicted pain worsening, joint space loss (JSL) and their combination (Kraus et al, 2017a). The odds of predicting case status were stronger comparing cases with 'pure' non-progressors (uCTX-II OR 1.72, uC2C-HUSA OR 1.50 based on 24M TIC) versus comparing cases with all comparators (uCTXII OR 1.37, uC2C-HUSA OR 1.27 based on 24 M TIC). Secondary analyses indicated that both uCTX-II and uC2C-HUSA were associated with all 3 progressor groups, pain-only worsening, JSL-only and their combination.

Earlier, Tamm et al (2014) reported positive correlations of urine C2C-HUSA with symptoms as well as joint function. These correlations were strongest when C2C-HUSA was also expressed per creatinine.

The C2C-HUSA and CTXII urine immunoassays detect type II collagen cleavage in cartilage by different enzymes and in different sites. Whereas C2C-HUSA detects type II collagen cleavage by collagenases predominately in uncalcified cartilage (Wu et al, 2002; Dejica et al, 2012), and has very limited association with adult calcified articular cartilage (tidemark), the CTX-II cleavage neoepitope, which is not generated by primary cleavage by collagenases, is predominately localized in calcified cartilage, osteophytes and at the osteo-chondral junction (Bay-Jensen et al, 2008; Huebner et al, 2014)). Moreover, the uCTX-II biomarker is strongly associated with the intensity of bone scintigraphic uptake, reflective of bone turnover, with osteophyte severity and knee OA progression (based on osteophyte score) but not progression based upon joint space narrowing (Huebner et al, 2014). In contrast, C2C-HUSA is closely associated with knee OA progression based on joint space loss/narrowing (Poole et al, 2016). An earlier biomarker study also showed with principal component analyses that CTX-II clusters with biomarkers of bone turnover (van Spil et al, 2012).

In further analyses (Nelson et al, 2019) of publicly available data from the FNIH/OARSI Biomarkers Consortium using a machine learning approach to identify biomarker "phenotypes" of progressors versus non-progressors of knee OA, C2C and C1,2C were analyzed in serum and C2C-HUSA (mislabeled as urine C2C) per creatinine was used to assay urine. Here, unlike CTX-II, none of these 3 assays recognized phenotypic differences in patients on comparative analysis and thereby *distinguish* between progressors and non-progressors. Yet we know that the urine based C2C-HUSA assay can *predict* subsequent progression in patients (Poole et al, 2016; Kraus et al, 2017a). This might suggest that differences in calcified cartilage turnover offer a phenotypic distinction whereas uncalcified cartilage does not. But importantly, both biomarkers can independently predict progression.

Earlier studies with COMP showed that mechanically-induced biomarker changes in patients with ACL injury or following ACL reconstruction can be used to identify patients at risk for progression to OA measured as regional changes in knee articular cartilage thickness 5 years later (Chu et al, 2018). To investigate whether other biomarkers can be used, subjects were again exercised on a treadmill for 30 min. after taking baseline blood samples at rest prior to exercise. Serum samples were taken again 5.5 hours after exercise. MRIs of the index knees were acquired at baseline and after 5 years. Changes in serum C1,2C and CS846 (from at rest to 5.5h) correlated with and were predictive of changes in articular cartilage thickness in specific regions detected 5 years later. For knees where the catabolic and anabolic marker concentrations increased, specific regions of articular cartilage were thinner 5 years later. The study supports the hypothesis that a mechanical stimulus to knee function can enable detection of changes in both markers of degeneration and synthesis that correlate with subsequent increases in articular cartilage thickness, in this case reflective of decreased cartilage matrix integrity. Many of the thickness changes were in the lateral compartment of both the tibia and femur. Given that the patients in this study had 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. The thickening of the cartilage is often misinterpreted as a sign of repair when, in fact, it is due to the established propensity of diseased articular cartilage's tendency to initially swell, caused by damage to the collagen fibrillar network permitting increased hydration of aggrecan and tissue swelling (Bank et al, 2000). This is followed by cartilage thinning. These important findings support further exploration of this approach for predicting OA progression.

Additional studies by this group (Titchenal et al, 2018) examined knee loading during walking and compositional MRI. CS846 changes correlated with both UTE-T2* (increase of which indicates early cartilage degradation) in all regions of deep weight bearing cartilage analyzed and with knee loading during walking. But there were no significant correlations between C1,2C response and MRI or knee loading.

6.3. Knee and hip OA haplotypes: When knee or hip OA patients were haplotyped for mitochondrial haplogroups, C2C, CPII and the C2C:CPII ratio were significantly increased in sera of OA patients carrying haplogroup H compared to OA carriers of the J haplogroup (Fernandez-Moreno et al, 2012). Collagenase MMP-13 is also more elevated in sera of 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 articular cartilage degeneration in knee OA (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 the OA is bilateral, unilateral or present in multiple sites (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. See also the work of Bender et al (2019) on CPII in hand OA with Bouchard's nodes.

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. Biomarkers to study post-traumatic knee OA

Direct analyses were made of articular cartilage removed at surgery for ACL repair less than a year after injury (Nelson et al, 2006). These have revealed increases in glycosaminoglycan content (per cell/DNA) and increased cleavage (C1,2C) and denaturation of type II collagen.

Arthroscopic analyses of pre-radiographic knee cartilage degeneration following ACL injury have revealed significant associations of increased SF C2C with the presence of 3 or more high Outerbridge-graded focal cartilage lesions (Yoshida et al, 2013).

Increases in knee SF C2C levels occured within a day following various ACL injuries, including ACL damage, and persisted up to 7 years (Kumahashi et al, 2015). Interestingly, C2C concentrations in SF and serum were correlated.

CS846 epitope is increased in knee SF of all study groups of patients with joint injuries compared with the uninjured reference group, being highest in patients with primary OA. (Lohmander et al, 1999). It is also positively correlated with SF COMP and CPII.

In a study of SF from patients with recent knee injuries and OA (Huang et al, 2018), FRZB levels (reflective of Wnt signalling) in the injury group correlated with C2C as well as COMP and the ARGS-aggrecan cleavage site. In contrast, DKK1 (Wnt signalling inhibitor) and GREM1 (bone morphogenetic protein pathway antagonist) were inversely correlated with these biomarkers pointing to the involvement of these regulatory molecules in joint pathology.

Biomarker analyses following knee ACL rupture, in which baseline serum pre-injury samples were available, showed subsequent serum changes of C2C, C1,2C, CPII and CS846 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. Although no pre-injury differences in the ratio of C1,2C:CPII or C2C:CPII were observed between groups, post-injury differences were observed for both ratios.

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 provided 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 when comparing the low and high-risk groups for OA onset (Wasilko et al, 2016).

Patients with a reconstructed ACL (Pietrosimone et al, 2016a) have lower serum C2C:CPII ratios associated with higher peak vertical ground-reaction force (vGRF) in the injured limb. Reduced vGRF limb symmetry indices are associated with higher C2C:CPII ratios after ACL injury but this was not significant after accounting for walking speed (Pietrosimone et al, 2017). These observations suggest that 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 may be increased damage to articular cartilages of these individuals. The same group found that the greater the time between injury and ACL reconstruction was associated with higher C2C:CPII ratios in males but not females at 6 months following reconstruction (Davis et al, 2018). These gender differences are of interest in view of the known disproportionate higher incidence of non-contact ACL injury in females.

A comparison of SF and serum levels of C1,2C and CS846 in patients with degenerative meniscal tears (DMT) and ACL damage (Brown et al, 2013) revealed significantly higher mean levels of both biomarkers in SF of ACL injured patients. Serum levels of C1,2C were higher in DMT patients whereas serum CS846 was lower in the ACL cohort than in the DMT cohort. No correlations were reported between SF and serum values for a given biomarker or between biomarkers.

8. Biomarkers to predict 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 on entry to West Point prior to sustaining ACL injury. In another study of young athletes who suffered shoulder injury and instability, pre-injury 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.

9. Biomarkers to monitor treatment of people with and without osteoarthritis

The use of biomarkers in the development of treatments for arthritis and monitoring their use in patients 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 9 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 FNIH and industry. Out of 18 assays examined the

C2C-HUSA urine assay was one 5 identified as of importance in future studies: there was another assay, urine CTX-II, mainly for calcified cartilage and the urine CTX-I and NTX-I assays for bone resorption and serum hyaluronan for synovitis (Kraus et al, 2016a). For more details see section 6.2/Progression above.

There have been 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 knee OA whereby glucosamine sulfate was administered 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 subsequent study found that CTX-II ($P<0.01$) and CPII ($P=0.08$) levels were substantially elevated in soccer players compared with those in controls, indicating that cartilage metabolism is increased in soccer players. Glucosamine administration over 3 months significantly decreased the CTX-II levels but was without any effect on CPII (Yoshimura et al, 2009). Later, Tsuruta et al (2018) conducted a similar study in collegiate soccer players. Urine CTX-II and serum C2C were both reduced compared to the placebo group. CPII was also measured but in urine. Since the assay was never developed and previously used for urine detection one must question the significance of any results for this body fluid. Tomonaga et al (2016) have observed that N-acetyl-glucosamine administration in healthy individuals suppressed the C2C/PIICP ratio compared to control groups. Yet N-acetylglucosamine administered over 16 weeks to persons without symptomatic or radiographic evidence of OA revealed no effect on serum C2C and CPII (Kubomura et al, 2017). A study of the effects of glucosamine hydrochloride on bicycle racers revealed no effect on CPII but CTX-II and the CTX-II/CPII ratio were reduced in a dose dependent manner (Momomura et al, 2013). A 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. Thus, there is evidence to indicate a suppression of type II collagen degradation in this sub-group and in some other studies.

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 whereas few a 15-25% of patients exhibit 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 there was a small increase 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 3 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.

A study of beneficial effects of exercise therapy in patients with no or early knee OA, namely KL≤1 and KL≥2 respectively, revealed that urine C2C was significantly reduced when both groups were analyzed together and reduced, but not significantly, when analyzed separately (Azukizawa et al, 2019). Serum PIICP was also measured and it increased when measured in the combined groups and only in the

KL≤1 when examined individually. Unfortunately, serum C2C was not measured and no attempt was made to relate, by ratios, degradation to synthesis of type II collagen.

A recent systematic review and meta-analysis of 12 randomized clinical trials by Bricca et al (2019) examined the impact of exercise therapy on molecular biomarkers related to cartilage and inflammation in people at risk for or with established knee OA. The therapy was associated with reductions of C2C in 30% of trials along with reductions in several cytokines, COMP and CTX-II: no increases were noted suggesting non-harmful effects of therapy.

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

10. Biomarker studies of spinal pathology

To date this is a relatively unexplored area with biomarkers. But an examination of patients with degenerative lumbar scoliosis (Hosogane et al, 2012) revealed that whereas serum HA and COMP showed no differences compared to controls, both serum CPII and C2C are significantly elevated in 52 patients when there is a Cobb angle of $>10^0$. Moreover, there was a significant positive correlation between the Cobb angle and CPII.

In 547 patients, as part of the Johnston County OA study (Goode et al, 2012), significant differences in mean biomarker levels were found across severity of disc space narrowing (DSN) for lnHA and lnC2C: lnCTX-II differences related to severity of both DSN and osteophytes. Moderate-to-strong associations were found between biomarkers of type II collagen and DSN, whereas associations with OST were weak.

11. Biomarkers to study osteochodrosis and osteochondritis dissecans (OCD)

Osteochondrosis is a developmental derangement of normal bone growth, primarily involving the centers of ossification in the epiphysis. Osteochondritis dissecans is another form of osteochondrosis. It occurs when small pieces of cartilage and bone become dislodged in the joint. IBEX Biomarkers have recently been shown to be of value in studying the ability of osteochondral scaffolds to repair focal lesions in patients with OCD. A prognostic prospective study revealed that both serum C2C and CPII, and ratios thereof, can be used to identify clinical success as measured by the International Knee Documentation Committee (IKDC) score (Gabusi et al, 2018a). This approach offers a new sensitive tool with which to monitor OCD patients over time during follow-up. Another study found that the histological cartilage tissue scores of osteochondral fragments correlated with patients' C2C and CPII biomarkers. CPII also correlated with histological bone tissue score (Gabusi et al, 2018b).

12. Biomarkers for animal studies including 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 identical or almost homologous sequences in epitopes across species has enabled their use in a number of animal biomarker studies. Some of these are described here.

12.1. Equine

Analyses of cartilage degradation in culture: When explants of equine articular cartilage are cultured *in vitro* progressive cleavage of type II collagen can be observed by C2C assay of culture media (Noe et al, 2017). This is much increased in OA explants compared to healthy cartilage. Stimulation of healthy cartilages by a combination of IL-1β or TNFα plus oncostatin M leads to an increase in type II collagen cleavage.

Osteochondrosis (OCD) and osteochondritis: 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).

Chavez et al (2016) determined serum and synovial concentrations of CS-846 in horses with a radiological diagnosis of osteochondritis. Twenty thoroughbred horses with unilateral radiocarpal or intercarpal OF were used, and 10 clinically and radiologically healthy Thoroughbred horses were assigned to the control group. Serum and SF concentrations of the CS846 epitope were measured. SF CS846 was significantly higher in carpal joints with moderate articular damage (grade 2 OF) than in the control group or in horses with severe disease (grade 3 and 4 OF). In serum, non-significant differences in CS846 epitope were observed in OF versus controls.
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.

Intra-articular steroids and anesthetics: 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 intra-articular 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).

Using a saline control, a single intra-articular injection of liposomal bupivacaine increased synovial fluid C1,2C and C2C after 96 hr as well as an increase in CPII at 48 hr. and CS846 at 24 and 48 hr. (Knych et al, 2019). As a liposomal control was not used it remains to be seen whether the effect is due to bupivacaine alone.

Osteochondral (OC) and other musculoskeletal 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 radiocarpal and intercarpal joints, SF analyses of CS846 revealed increases in injured joints compared to controls whereas CPII showed no changes. The CS846 changes correlated with the severity of injury based on arthroscopic and radiographic scores. Serum CS846 and CPII concentrations were both significantly higher in horses with OC injuries than in control horses. Discriminant analysis allowed 27 of 34 (79%) horses to be correctly classified as having OC based on serum CS846 and CPII concentrations.

Trumble et al (2009) found that SF C2C concentrations in OC injured joints of Thoroughbreds were also positively correlated with radiographic and arthroscopic scores, both of the latter clinical measures being positively correlated positively correlated with each other. SF C2C concentrations for individual joints discriminated OC injured joints from rested or exercised joints.

In foals with tarsocrural OCD the CPII:C2C ratio in SF tended to be higher in affected joints of foals relative to controls at all ages and significant at 22 weeks of age: CS846 was reduced at 18 weeks (de Grauw et al, 2011).

Osteoarthritis: In early experimental OA in horses, SF levels of C1,2C, CPII and CS846 are all increased (Frisbie et al, 2008). A study of OA in an older (>8 years) horse population employing radiographs, MRI arthroscopy, gross morphology and SF biomarkers (Coppelman, 2017) found tibial tarsal joint C2C and IL-6 best reflected OA severity. The best for OA severity in the distalintertarsal joint included CPII and C2C. The latter also correlated with subchondral bone hyperintensity on MRI. In tarso-metatarsal joints CPII was the best biomarker reflecting OA severity. It was concluded that biomarkers have the potential to be a valuable source of insight into OA pathology in tarsal joints.

12.2. Canine

Induction of unilateral OA in dogs by ACL transection leads to elevations in SF C2C. Starting at 3 weeks it is 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 canine OA caused by experimental damage to the cranial cruciate ligament (Chu et al, 2002). Concentrations of SF CS846 exhibit a non significant increase.

The natural development of OA in dogs with medial coronoid disease with dysplastic elbows is accompanied by an increase in SF C2C (Prink et al, 2010). This is correlated with the degree of cartilage damage. Earlier work by this group failed to reveal any changes in C2C in serum, urine and SF in dogs with cranial cruciate ligament rupture that often leads to OA (Hayashi et al, 2009).

As mentioned previously, 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.

12.3. Murine

Murine OA in biglycan/fibromodulin double-deficient mice, 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 wild type mice (Stoop et al, 1999). Similar results were observed for cartilage degradation in antigen-induced inflammatory arthritis (van Meurs et al 1999).

12.4. Rat

Intra-articular injection of streptococcal cell walls creates an inflammatory arthritis in rats. This results in a serum C2C increase with onset of acute inflammation which rises further in chronic disease. This increase was much reduced on effective 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).

In a rat OA model involving ACL transection, serum COMP and CS846 both increased as joint degeneration developed. The biomarkers correlated with each other and with OARSI scores of joint degeneration (Ma et al, 2018).

12.5. 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 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 ACL transection results in the elevation of SF C2C (Wei et al, 2010).

12.6. Porcine

Compared with unaffected pigs, serum CPII is significantly increased and C2C is significantly decreased in pigs with osteochondrosis (Frantz et al, 2010). A 2-fold rise in CPII increases the odds of pigs having OC by a factor of 97.

MRI studies of healthy animals and those with naturally occurring OA (Hatcher et al, 2017) showed 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.

12.7. Lapine

Rabbits with antigen (ovalbumin) induced monoarticular inflammatory arthritis also show an increase in SF C2C in the affected stifle joint, but not in serum (Kojima et al, 2001).

12.8. Elephant

In chronically lame Asian elephants, serum CPII and C2C are decreased (Kilgallon et al, 2015).

13. Concluding remarks

This updated and expanded overview of research involving IBEX antibodies and biomarkers reveals their widespread use and value. This is especially apparent for the molecular biomarkers in basic and clinical research. Along with other biomarkers, they are helping improve 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 OA, such as knee OA, is not always a continuously progressive disease.

The potential offered by biomarkers such as C2C-HUSA in clinical trials, on a virtual par with CTX-II in its clinical value. Yet, as we have seen, unlike CTX-II, C2C-HUSA is specific for cartilage erosion. Moreover, analyses of a control "non-OA" population has revealed that unlike urine C2C-HUSA, the mean concentration of urine CTX-II is higher in women than in men and higher in African Americans compared to Caucasians (Kraus et al, 2017b).

The use of such biomarkers also enables the identification of sub-groups with active disease. This will be of great importance in identifying "progressor" patients for inclusion 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).

An area where these biomarkers have received little clinical application is in the *in vivo* study of skeletal growth and development, its impairment and treatment. The little work that has been done has proved to be very informative. This is an area where important opportunities remain for their use. I would have liked to pursue this area much more but I was unsuccessful in gaining access to populations to conduct such studies.

What is so rewarding to see today is the commitment to well-structured programs to create independent assessments of new biomarker assays and their applications. In the arthritis field this is still in its infancy, started by the foresight of people such as Joan McGowan (National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIAMS) who, with her colleagues, led the creation of the OA Initiative in 2000, a private/public initiative. This was when she invited me to write the Biomarker White Paper. Others wrote similar position papers on imaging and clinical studies of OA with which the Initiative and founding conference were launched. Many years later OARSI got involved as well as FNIH to work with the FDA to launch the independent assessment phase for existing biomarkers. This Initiative has already provided an invaluable resource for many important studies. It is very unfortunate that this momentum appears to have stalled of late, apparently a result of insufficient funding to complete the molecular/biochemical biomarker assessment for the FDA. This is

of concern as so much valuable work has already been done. Let's hope that there is a quick solution to this problem.

As I and many others have shown, these biomarkers have much value in studying the physiology and pathology of joint and other tissues directly, including calcified and other cartilages and tissues rich in type I collagen (degradation of latter detected with biomarker C1,2C). These type I rich tissues include the majority of the tissues within the body from which type II collagen is absent, only in the posterior chamber of the eye is type II collagen also ordinarily found.

I hope that this review 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.

14. References

1. Ameye LG, Deberg M, Oliveira M, Labasse A, Aeschlimann JM, Henrotin Y. (2007). The chemical biomarkers C2C, Coll2-1 and Coll2-1NO2 provide complimentary information on type II collagen catabolism in healthy and osteoarthritic mice. *Arthritis Rheum.* 56: 3336-46.

2. Antoniou J, Steffen T, Nelson F, Winterbottom N, Hollander AP, et al. (1996). The human lumbar intervertebral disc. Evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, ageing and degeneration. *J. Clin. Invest.* 98: 996-1003.

3. Armstrong L, Thickett DR, Mansell JP, Ionescu M, Hoyle E, et al. (1999). Changes in collagen turnover in early acute respiratory distress syndrome. *Am. J. Resp. Critical Care. Med.* 160: 1910-15.

4. Aurich M, Squires GR, Reiner A, Mollenhauer JA, Keuttner KE et al (2005). Differential matrix degradation and turnover in early cartilage lesions of human Knee and Ankle Joints. *Arthritis Rheum*. 52(1):112-119.

5. Azukizawa M, Ito H, Hamamoto Y, Fujii T, Morita Y et al. (2019). The effects of well-rounded exercise program on sysytemic biomarkers related to cartilage metabolism. *Cartilage.* 10: 451-8.

6. Bank RA, Soudry M, Maroudas A, Mizrahi J, TeKoppele JM. (2000). The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. *Arthritis Rheum.* 43: 2202-10

7. Bay-Jensen AC, Andersen TL, Charni-Ben Tabassi N, Kristensen PW et al. (2008). Biochemical markers of type II collagen breakdown and synthesis are positioned at specific sites in human osteoarthritic knee cartilage. *Osteoarthritis Cartilage.* 16; 615-23.

8. Bender A, Kaessner U, Eichner G, Bachmann G, Steinmeyer J. (2019). Biomarkers of hand osteoarthritis are detectable after mechanical exercise. *J. Clin. Medicine.* 8:1545.

9. Billinghurst RC, Brama PA, van Weeren PR, Knowlton MS, McIlwraith CW. (2004). Evaluation of serum concentrations of biomarkers of skeletal metabolism and results of radiography as indicators of severity of osteochondrosis in foals. *Am. J. Vet. Res.* 65:143-50.

10. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R et al. (1997). Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J. Clin. Invest.* 99:1534-45.

11. Boeth H, MacMahon A, Poole AR, Buttgereit F, Onnefjord P et al. (2017). Differences in biomarkers of cartilage matrix turnover and their changes over 2 years in adolescent and adult volleyball athletes. *J. Exp. Orthop.* 4: 1-11.

12. Bricca A, Struglics A, Larsson S, Steultjens M, Juhl CB, Roos EM. (2019). Impact of exercise therapyon molecular biomarkers related to cartilage and inflammation in people at risk of, or with established, knee osteoarthritis: a systematic review and meta-analysis of randomized controlled trials. *Arthritis Care Res* (Hoboken). 71: 1504-15.

13. Briot K, Roux C, Gossec L, Charni N, Kolta S et al. (2008). Effects of etanercept on serum biochemical marker of cartilage metabolism in patients with spondyloarthropathy. *J. Rheumatol.* 35:310-14.

14. Brown CW, Huang CH, Stepp PC, Chu CR. (2013). Serum and synovial fluid biomarkers of cartilage and matrix turnover in human subjects with anterior cruciate ligament and degenerative meniscal tears. *Trans. Orthop. Res. Soc.* 483.

15. Cahue S, Sharma L, Dunlop D, Ionescu M, Song J. et al. (2007). The ratio of type II collagen breakdown to synthesis and its relationship with the progression of knee osteoarthritis. *Osteoarthritis Cartilage.* 15: 819-23.

16. Carey DE, Alini M, Ionescu M, Hyams JS, Rowe JC, et al. (1997). Serum content of the C-propeptide of the cartilage molecule type II collagen in children. *Clin. Exp. Rheumatol.* 15: 325-8.

17. Celeste C, Ionescu M, Poole AR, Laverty S. (2005). Repeated intaarticular injections of triamcinolone acetonide alter cartilage matrix metabolism measured by biomarkers in synovial fluid. *J. Orthop. Res.* 23:602-10.

18. Chandran V, Cook RJ, Edwin J, Shen H, Pellett FJ et al. (2010). Soluble biomarkers differentiate patients with psoriatic arthritis from those with psoriasis without arthritis. *Rheumatology.* 49:1399-405.

19. Chavez H, Folch H, Araya O, Uberti H, Moran G. (2016). Concentration of the CS-846 Epitope in Serum and Synovial Fluid of Horses with Different Grades of Osteochondral Fragments in the Carpal Joints. *Gen. Med.* 4:3 DOI: 10.4172/2327-5146.1000242

20. Chu CR, Sheth S, Erhart-Hledik JC, Do B, Titchenal, MR, Andriacchi, TP. (2018). Mechanically stimulated biomarkers signal cartilage changes over 5 years consistent with disease progression in medial knee osteoarthritis patients. *J. Orthop. Res.* 36:891-7.

21. Chu Q, Lopez M, Hayashi K, Ionescu M, Billinghurst RC et al. (2002). Elevation of a collagenase generated type II collagen neoepitope amd a proteoglycan epitope in synovial fluid following induction of joint instability in the dog. *Osteoarthritis Cartilage.* 10: 662-9.

22. Cibere J, Thorne A, Kopec JA, Singer J, Canvin J, et al. (2005). Glucosamine sulfate and cartilage: type II collagen degradation in patients with knee osteoarthritis: randomized discontinuation trial results employing biomarkers. *J. Rheumatol.* 32: 896-902.

23. Cibere J, Zhang H, Garnero P, Poole AR, Lobanok T. et al. (2009). Association of biomarkers with with pre-radiographically defined and radiographically defined knee osteoarthritis in a population-based cohort. *Arthritis Rheum.* 60: 1372-80.

24. Conrozier T, Poole AR, Ferrand F, Mathieu P, Vincent F et al. (2008). Serum concentrations of type II collagen biomarkers (C2C, C1,2C and CPII) suggest different pathophysiologies in patients with hip osteoarthritis. *Clin. Exp. Rheumatol*. 26: 430-5.

25. Coppelman E. (2017). The use of biomarkers to determine the severity of osteoarthritis in the tarsus of an older horse population. Thesis submitted to the University of Minnesota.

26. Dahlberg L, Billinghurst C, Manner P, Ionescu M, Reiner A et al. (2000). Selective enhancement of collagenase-mediated cleavage of resident type II collagen in cultured osteoarthritic cartilage and arrest with a synthetic inhibitor that spares collagenase 1 (matrix metalloproteinase 1). *Arthritis Rheum.* 43: 673-82.

27. Davis HC, Spang JT, Loesser RF, Larsson S, Ulici V et al. (2018). Time between anterior cruciate ligament injury and reconstruction and cartilage metabolism six-months following reconstruction. *Knee.* 25:296-305.

28. de Grauw JC, Donabedian M, van der Lest CH, Perona G, Robert C et al. (2011). Assessment of synovial fluid biomarkers in healthy foals and in foals with tarsocrural osteochondrosis. *Vet. J.* 190:390-5.

29. Dejica V, Mort JS, Laverty S, Antoniou J, Zukor DJ et al. (2012). Increased type II collagen cleavage by cathepsin K and collagenase activities with aging and osteoarthritis in human articular cartilage. Arthritis Res Therapy 14: R113. 30.Dejica VM, Mort JS, Laverty S, Percival MD, Antoniou J et al. (2008). Cleavage of type II collagen by cathepsin K in human osteoarthritis cartilage. *Am. J. Pathol.* 173: 161-9.

31. Donabedian M, van Weeren PR, Perona G, Fleurance G, Robert C et al. (2008). Early changes in biomarkers of skeletal metabolism and their association to the occurrence of osteochondrosis (OC) in the horse. *Equine Vet. J.* 40:253-9.

32. Engstrom-Laurent A. (1989). Changes in hyaluronan concentration in tissues and body fluids in disease states. In: The Biology of Hyaluronan. D. Evered, J. Whelan (Eds.), John Wiley and Sons, Chichester, UK, pp. 233-247.

33. Fernandez-Moreno M, Soto-Hermida A, Oreiro N, Pertega S, Fenandez-Lopez C et al. (2012). Mitochondrial haplogroups define two phenotypes of osteoarthritis. *Front. Physiol.* 3:129

34. Frantz NZ, Friesen KG, Andrews GA, Tokach MD, Yamka RM et al. (2010). Use of serum biomarkers to predict the development and severity of osteochondrosis lesions in the dital portion of the femur in pigs. *Am. J. Vet. Res.* 71: 946-52

35. Fraser A, Fearon U, Billinghurst RC, Ionescu M, Reece R et al. (2003). Turnover of type II collagen and aggrecan in cartilage matrix at the outset of inflammatory arthritis in humans. *Arthritis Rheum.* 48: 3085-95.

36. Frisbie DD, Al-Sobayil F, Billinghurst RC, Kawcak CE, McIlwraith CW. (2008). Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. *Osteoarthritis Cartilage.* 16:1196-2004.

37. Frisbie DD, Ray CS, Ionescu M, Poole AR, Chapman PL, McIlwraith CW. (1999). Measurement of synovial fluid and serum concentrations of the 846 epitope of chondroitin sulfate and of carboxy peptides of type II procollagen for diagnosis of osteochondral fragmentation in horses. *Am. J. Vet. Res.* 60:306-9.

38. Gabusi E, Manferdini C, Paolella F, Gambari L, Kon E et al. (2018b). Clinical and Biological signature of osteochondritis dessicans in a cross-sectional study. *Biomed. Res. Int.* 2018: 5458704

39. Gabusi E, Paolella F, Manferdini C, Gambari L, Kon E et al. (2018a). Cartilage and bone serum biomarkers as novel tools for monitoring knee osteochondritis dissecans treated with osteochondral scaffold. *Biomed. Res. Int.* 2018: 9275102.

40. Goode AP, Marshall SW, Kraus VB, Renner JB, Stürmer T et al. (2012). Associations between serum and urine biomarkers and lumbar spine individual radiographic features: the Johnston County osteoarthritis project. *Osteoarthritis Cartilage.* 20: 1286-93.

41. Hatcher CC, Collins AT, Kim SY, Michel LC, Mostertz WC 3rd et al. (2017). Relationship between T1rho magnetic resonance imaging, synovial fluid biomarkers, and the biochemical and biomechanical properties of cartilage. *J. Biomech.* 55:18-26.

42. Hayashi PA, Kim SY, Lansdowne JL, Kapatkin A, Dejardin LM. (2009). Evaluation of a collagenase generated osteoarthritis biomarker in naturally occurring canine cruciate disease. *Vet. Surg.* 38: 1117-21

43. He G, Chen X, Zhang G, Lin H, Wu X. (2014). Detection of urine C2C and trace element level in patients with knee osteoarthritis. *Cell Biochem. Biophys.* 70:475-9.

44. Hosogane N, Watanabe K, Tsuji T, Miyamoto T, Ishii K et al. (2012). Serum cartilage metabolites as biomarkers of degenerative lumbar scoliosis. *J. Orthop. Res.* 30:1249-53

45. Huang X, Post JN, Zhong L, Leijten J, Larsson S et al (2018). Dickkopf-related protein 1 and gremlin 1 show different response than frizzled-related protein in human synovial fluid following knee injury and in patients with osteoarthritis. *Osteoarthritis Cartilage.* 26: 834-43.

46. Huebner JL, Bay-Jensen AC, Huffman KM, He Y, Leeming DJ et al (2014). Alpha C-telopeptide of type I collagen is associated with subchondral bone turnover and predicts progression of joint space narrowing and osteophytes in osteoarthritis. *Arthritis Rheumatol*. 2014 Sep;66(9):2440-9

47. Huebner JL, Kraus VB. (2006), Assessment of the utility of biomarkers of osteoarthritis in the guinea pig. *Osteoarthritis Cartilage*. 14:923-30.

48. Hunt MA, Pollock C L, Kraus VB, Saxne T, Peters S et al. (2013). Relationships amongst osteoarthritis biomarkers, dynamic knee joint load, and exercise: results from a randomized controlled pilot study. *BMC Musculoskeletal Disord.* 14:115.

49. Ishijima M, Watari T, Naito K, Kaneko H, Futami I et al. (2011). Relationships between biomarkers of cartilage, bone, synovial metabolism and knee pain provide insights into the origins of pain in early knee osteoarthritis. *Arthritis. Res. Therapy.* 13: R22

50. Jansen NW, Roosendaal G, Lundin B, Heijenen L, Mause-Bunschoten E et al. (2009). The combination of the biomarkers urinary C-terminal telopeptide of type II collagen, serum cartilage oligomeric matrix protein, and serum chondroitin sulfate 846 reflects cartilage damage in hemophilic arthropathy. *Arthritis Rheum.* 60: 290-8.

51. Kilgallon CP, Larsen S, Wong A, Yellowley C. (2015). Analysis of a collagen II degradation protein C2C and a collagen II formation protein CPII in serum of Asian elephants (Elephas maximus). *J. Zoo. Wild Led.* 46: 146-9.

52. Kim T-H, Stone M, Payne U, Zhang X, Ionescu M et al. (2005). Cartilage biomarkers in ankylosing spondylitis. Relationship to clinical variables and treatment response. *Arthritis Rheum.* 52: 885-91.

53. King KB, Lindsey CT, Dunn TC, Ries MD, Steinbach LS, Majumdar S. (2004). A study of the relationship between molecular biomarkers of joint degeneration and the magnetic resonance-measured characteristics of cartilage in 16 symptomatic knees. *Magn. Reson. Imaging.* 22: 1117-23.

54. Knych HK, Mama KR, Moore CE, Hill AE, McKemie DS. (2019). Plasma and synovial fluid concentrations and cartilage toxicity of bupivacaine following intra-articular administration of a liposomal formulation to horses. *Equine Vet.* 51:408-14.

55. Kojima T, Kojima M, Noda K, Ishiguro N, Poole AR. (2008). Influences of menopause, aging and gender on the cleavage of type II collagen in cartilage in relationship to bone turnover. *Menopause.* 15:133-7.

56. Kojima T, Mwale F, Yasuda T, Girard C, Poole AR, Laverty S. (2001). Early degradation of type IX and type II collagen with the onset of experimental arthritis. *Arthritis Rheum.* 44: 120-7.

57. Kong SY, Stabler TV, Criscione LG, Elliott AL, Jordan KM, Kraus VB. (2006). Diurnal variation of serum and urine biomarkers in patients with radiographic knee osteoarthritis. *Arthritis Rheum.* 54: 2496-504

58. Kraus VB, Burnett B, Coindreau J, Cottrell S, Eyre D et al. (2011). Application of biomarkers in the development of drugs intended for the treatment of osteoarthritis. *Osteoarthritis Cartilage.* 19: 515-42

59. Kraus VB, Collins JE, Hargrove D, Losina E, Nevitt M et al. (2017a) Predictive validity of biochemical biomarkers in knee osteoarthritis: data from the FNIH OA Biomarkers Consortium. *Ann Rheum Dis*. 76:186-95.

60. Kraus V, Hargrove DE, Hunter DJ, Renner JB, Jordan JM. (2017b). Establishment of reference intervals for osteoarthritis-related soluble biomarkers: the FNIH/OARSI biomarkers consortium. *Ann. Rheum. Dis*. 76:179-85

61. Kubomura D, Ueno T, Yamada M, Tomonaga M, Nagaoka I. (2017). Effect of N-acetylglucosamine administration on cartilage metabolism and safety in healthy subjects without symptoms of arthritis: A case report. *Exp. Ther. Med.* 13: 1614-21.

62. Kumahashi N, Sward P, Larsson S, Lohmander LS, Frobell R, Struglics A. (2015). Type II collagen C2C epitope in human synovial fluid and serum after knee injury-associations with molecular and structural markers of injury. *Osteoarthritis Cartilage*. 23: 1506-12.

63. Laverty S, Ionescu M, Marcoux M, Boure L, Doize B, Poole AR. (2000). Alterations in cartilage type II procollagen and aggrecan contents in synovial fluid in equine osteochondrosis. *J. Orthop. Res*. 18: 399-405.

64. Laverty S, O'Kouneff S, Ionescu M, Reiner A, Pidoux I et al (2002). Excessive degradation of type II collagen in articular cartilage in equine osteochondrosis. *J. Orthop. Res.* 20: 1282-9.

65. Leff RL, Elias I, Ionescu M, Reiner A, Poole AR. (2003). Molecular changes in human osteoarthritis cartilage after 3 weeks of oral administration of BAY12-9566, a matrix metalloproteinase inhibitor. *J. Rheumatol*. 30: 544-9.

66. Lettry V, Sumie Y, Mitsuda K, Tagami M, Hosoya K et al. (2010). Divergent diagnosis from arthroscopic findings and identification of CPII and C2C for detection of cartilage degradation in horses. *Japan J. Vet. Res*. 57: 197-206.

67. Lohmander LS, Ionescu M, Jugessur H, Poole AR. (1999). Changes in joint cartilage aggrecan after knee injury and in osteoarthritis. *Arthritis Rheum*. 42: 534-544.

68. Ma J, Sorsa T, Billinghurst R, Poole AR, Kitti U et al. (2003) Direct evidence of collagenolysis in chronic periodontitis. *J. Periodontal Res*. 38: 564-7.

69. Ma T, Zhang Z, Song X, Bai H, Li Y et al. (2018). Combined detection of COMP and CS846 biomarkers in experimental rat osteoarthritis: a potential approach for assessment and diagnosis of osteoarthritis. *J. Orthop. Surg. Res*. 13: 230

70. Maksymowych WP, Poole AR, Hiebert L, Webb A, Ionescu M et al. (2005). Etanercept exerts beneficial effects on articular cartilage biomarkers of degradation and turnover in patients with ankylosing spondylitis. *J. Rheumatol*. 32: 1911-7.

71. Manicourt D-H, Azria M, Mindeholm L, Thonar E J-M, Devogelaer J-P. (2006). Oral salmon calcitonin reduces Lequesne's algofunctional index scores and decreases urinary and serum levels of biomarkers of joint metabolism in knee osteoarthritis. *Arthritis Rheum*. 54: 3205-11.

72. Mansson B, Carey D, Alini M, Ionescu M, Rosenberg LC, Poole AR, Heinegard D, Saxne T. (1995). Cartilage and bone metabolism in rheumatoid arthritis. *J. Clin. Invest*. 95: 1071-1077.

73. Matyas JR, Atley L, Ioenescu M, Eyre DR, Poole AR. (2004). Analysis of cartilage biomarkers in the early phase of canine experimental osteoarthritis. *Arthritis Rheum*. 50: 543-52

74. Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA et al. (1996). Cloning, expression and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. *J. Clin. Invest*. 97: 761-8.

75. Momomura R, Naito K, Igarashi M, Watari T, Terakado A et al. (2013). Evaluation of the effects of glucosamine administration on biomarkers of cartilage and bone metabolism in bicycle racers. *Mol. Med. Rep.* 7: 742-6

76. Mullan RH, Matthews C, Bresnihan B, Fitzgerald O, King L et al. (2007). Early changes in serum type II collagen biomarkers predict radiographic progression at one year in inflammatory arthritis patients after biologic therapy. *Arthritis Rheum*. 56: 2919-28.

77. Mündermann A, Klenk C, Billich C, Nüesch C, Pagenstert G et al. (2017). Changes in cartilage biomarker levels during a transcontinental multistage footrace over 4486 km. *Am. J. Sports Med*. 45:2630-6.

78. Mwale F, Tchetina E, Wu W, Poole AR. (2002). The assembly and remodeling of the extracellular matrix in the growth plate in relationship to mineral deposition and cellular hypertrophy: an *in situ* study of collagens II and IX and proteoglycan. *J. Bone Min. Res*. 17: 275-83.

79. Nelson AE, Fang F, Arbeeva L, Cleveland RJ, Schwartz TA et al. (2019). A machine learning approach to knee osteoarthritis phenotyping: data from the FNIH Biomarkers Consortium. *Osteoarthris Cartilage.* 27: 994-1001.

80. Nelson F, Billinghurst RC, Pidoux I, Reiner A, Langworthy M, et al. (2006). Early post-traumatic osteoarthritis-like changes in human articular cartilage following rupture of the anterior cruciate ligament. *Osteoarthritis Cartilage.* 14:114-19

81. Nelson F, Dahlberg L, Laverty S, Reiner A, Pidoux I et al. (1998). Evidence for altered synthesis of type II collagen in patients with osteoarthritis. *J. Clin. Invest*. 102: 2115-25.

82. Nemirovskiy OV, Dufield DR, Sunyer T, Aggarwal P, Welsch DJ, Mathews WR. (2007). Discovery and development of a type II collagen neoepitope (TIINE) biomarker for matrix metalloproteinase activity: from in vitro to in vivo. *Annal. Biochem.* 361: 93-101.

83. Niehoff A, Bruggemann G-P, Zaucke F, Eckstein F, Bloch W et al. (2016). Long-duration space flight and cartilage adaptation: first results on changes in tissue metabolism. *Osteoarthritis Cartilage.* 24:S144-5

84. Niki Y, Takeuchi T, Nakayama M, Nagasawa H, Kurasawa T et al. (2012). Clinical significance of cartilage biomarkers for monitoring structural joint damage in rheumatoid arthritis patients treated with anti-TNF therapy. *PLoS One*. 7: e37447

85. Noe B, Poole AR, Mort JS, Richard H, Beauchamp G et al. (2017). C2K77 ELISA detects cleavage of type II collagen by cathepsin K in equine articular cartilage. *Osteoarthritis Cartilage*. 25:2119-26

86. Oldenburg J, Zimmermann R, Katsarou O, Zanon E, Kellermann E et al. (2016). Potential biomarkers of haemophilic arthropathy: correlations with compatible additive magnetic resonance imaging scores. *Haemophilia*. 22: 760-4.

87. Otterness IG, Swindell AC, Zimmerer RO, Poole AR, Ionescu M, Weiner E. (2000). An analysis of 14 molecular markers for monitoring osteoarthritis: segregation of the 14 markers into clusters and distinguishing osteoarthritis at baseline. *Osteoarthritis Cartilage*. 8: 180-5.85.

88. Owens BD, Cameron KL, Bokshan SL, Clifton KB, Svoboda SJ, Wolf, JM. (2017). Serum cartilage biomarkers and shoulder instability. *Orthopedics.* 40: 34-6.

89. Pietrosimone B, Blackburn JT, Harkey MS, Luc BA, Hackney AC et al. (2016a). Greater mechanical loading during walking is associated with less collagen turnover in individuals with anterior cruciate ligament reconstruction. *Am. J. Sports. Med*. 44:425-32.

90. Pietrosimone B, Blackburn T, Harkey MS, Luc BA, Hackney AC et al. (2016b). Walking speed as a potential indicator of cartilage breakdown following anterior cruciate ligament reconstruction. *Arthritis. Care. Res* (Hoboken). 68: 793-800.

91. Pietrosimone B, Loeser RF, Blackburn JT, Padua DA, Harkey MS, et al. (2017). Biochemical markers of cartilage metabolism are associated with walking biomechanics six-months following anterior cruciate ligament reconstruction. *J. Orthop. Res*. 35:2288-97.

92. Poole, A.R. (2000). NIH White Paper: Biomarkers, the Osteoarthritis Initiative. National Institute of Health, NIAMS News and Events. www.nih.gov/niams/news/oisg.

93. Poole AR (2016). Current opinion: where are we in our understanding and treatment of osteoarthritis? *Swiss Med. Wkly*. 146: w14340.

94. Poole AR, Ha N, Bourdon S, Sayre EC, Guermazi A, Cibere J. (2016). Ability of a urine assay of type II collagen cleavage by collagenases to detect early onset and progression of articular cartilage degeneration: Results from a population-based cohort study. *J. Rheumatol*. 43:1864-70.

95. Poole AR., Ionescu M, Fitzcharles MA, Billinghurst R. (2004). The assessment of cartilage degradation *in vivo*: Development of an immunoassay for the measurement in body fluids of type II collagen cleaved by collagenases. *J. Immunol. Methods*. 294:145-53.

96. Poole AR, Ionescu M, Swan A, Dieppe PA. (1994). Changes in cartilage metabolism in arthritis are reflected by altered serum and synovial fluid levels of the cartilage proteoglycan aggrecan. Implications for pathogenesis. *J. Clin. Invest*. 94: 25-33.

97. Prink A, Hayashi K, Kim SY, Kim J, Kapatkin A. (2010). Evaluation of a collagenase generated osteoarthritis biomarker in the synovial fluid from elbow joints of dogs with medial coronoid disease and unaffected dogs. *Vet. Surg*. 39: 65-70.

98. Ramonda R, Lorenzin M, Modesti V, Campana C, Ortolan A, et al. (2013). Serological markers of erosive hand osteoarthritis. *Eur. J. Intern. Med*. 24:11-5

99. Richette P, Eymard RP, Deberg M, Vidaud D, de Kerguenec C et al. (2010). Increase in type II collagen turnover after iron depletion in patients with hereditary haematochromatosis. *Rheumatology* (Oxford). 49: 760

100. Rizkalla G, Reiner A, Bogoch E, Poole AR. (1992). Studies of the articular cartilage proteoglycan in health and osteoarthritis: Evidence for molecular heterogeneity and extensive molecular changes in disease. *J. Clin. Invest*. 90: 2268-77.

101. Robion FC, Doize B, Boure L, Marcoux M, Ionescu M et al. (2001). Use of synovial fluid markers of cartilage synthesis and turnover to study effects of repeated intra-articular administration of methyl prednisolone acetate on articular cartilage. *J. Orthop. Res*. 19: 250-258.

102. Sigward SM, Pollard CD, Powers CM. (2012). The influence of sex and maturation on landing biomechanics: implications for ACL injury. *Scand. J. Med. Sci. Sports*. 22: 502-9.

103. Song X-Y, Zeng L, Jin W, Thompson J, Mizel DE et al. (1999). Secretory leucocyte protease inhibitor suppresses the inflammation and joint damage of bacterial cell wall-induced arthritis. *J. Exp. Med.* 190: 535-42.

104. Soto-Hermida A, Fernandez-Moreno M, Pertaga-Diaz S, Oreiro N, Fernando-Lopez C et al. (2015) Mitochondrial DNA haplogroups modulate the radiographic progression of Spanish patients with osteoarthritis. *Rheumatol. Int*. 35: 337-44.

105. Squires G, Okouneff S, Ionescu M, Poole AR. (2003). Pathobiology of focal lesion development in aging human articular cartilage reveals molecular matrix changes characteristic of osteoarthritis. *Arthritis Rheum*. 48: 1261-70.

106. Stoop R, Buma P, van der Kraan P, Hollander AP, Billinghurst RC et al. (2001). Type II collagen degradation in articular fibrillation and anterior cruciate ligament transection in rats. *Osteoarthritis Cartilage*. 9: 308-15.

107. Stoop R, van der Kraan PM, Buma P, Hollander AP, Billinghurst RC, et al. (1999). Type II collagen degradation in spontaneous osteoarthritis in C57B1/6 and BALB/c mice. *Arthritis Rheum*. 42: 2381- 9.

108. Struglics A, Saleh R, Sundberg E, Olsson M, Erlandsson Harris H, Aulin C. (2019). Juvenile idiopathic arthritis patients have a distinct cartilage and bone biomarker profile that differs from healthy and knee-injured children. *Clin. Exp. Rheumatol*. Epub, In press.

109. Svoboda SJ, Harvey TM, Owens BD, Brechue WF, Tarwater PM, Cameron KL. (2013). Changes in serum biomarkers of cartilage turnover after anterior cruciate ligament injury. *Am. J. Sports Med*. 41: 2108-16.

110. Svoboda, SJ, Owens, BD, Harvey TM, Tarwater,PM, Brechue WF Cameron, KL. (2016). Biomarkers of collagen turnover and subsequent anterior cruciate ligament rupture. *Am. J. Sports Med*. 44: 1687-93

111. Tamm AO, Kumm J, Tamm A, Lintrop M, Kukner A et al. (2014). Cartilage collagen neoepitope C2C and clinical parameters in middle-aged patients with knee problems. Correlations of urinary output of C2C with cartilage lesions, KOOS values and functional abilities of lower limb. *Osteoarthritis Cartilage.* 22: S70-S71.

112. Titchenal MR, Williams AA, Asay JL, Migliore E, Erhart-Hledik JC et al. (2018). Mechanically stimulated CS846 correlates with ultrashort echo time enhanced $T2^*$ quantitative MRI and gait mechanics 2 years after anterior cruciate ligament reconstruction. *Osteoarthritis Cartilage.* 26:344.

113. Tomonaga A, Takahashi T, Tanaka YT, Tsuboi M, Ito K, Nagaoka I. (2017). Evaluation of the effect of salmon nasal proteoglycan on biomarkers for cartilage metabolism in individuals with knee joint discomfort: A randomized double-blind placebo-controlled clinical study. *Exp. Ther. Med*. 14:115-26.

114. Tomonaga A, Watanabe K, Fukagawa M, Suzuki A, Kurokawa M, Nagaoka I. (2016). Evaluation of the effect of N-acetyl-glucosamine administration on biomarkers for cartilage metabolism in healthy individuals without symptoms of arthritis: A randomized double-blind placebo-controlled study. *Exp. Ther. Med*. 12: 1481-9.

115. Tourville TW, Johnson RJ, Slauterbeck JR, Naud S, Beynnon BD. (2013). Relationship between markers of type II collagen metabolism and tibiofemoral joint space width changes after ACL injury and reconstruction. *Am. J. Sports Med*. 41:779-87.

116. Trumble TN, Scarborough AB, Brown MP. (2009). Osteochondral injury increases type II collagen degradation products (C2C) in synovial fluid of Thoroughbred racehorses. *Osteoarthritis Cartilage*. 17:371-4.

117. Tsuruta A, Horiike T, Yoshimura M, Nagaoka I. (2018). Evaluation of the effect of the administration of a glucosamine-containing supplement on biomarkers for cartilage metabolism in soccer players: a randomized double-blind placebo-controlled study. *Mol. Med. Rep*. 18: 3941-8.

118. Tyler JA. (1985). Articular cartilage cultured with catabolin (pig interleukin 1) synthesizes a deceased number of normal proteoglycan molecules. *Biochem. J.* 227: 869-78.

119. Tyler JA, Benton HP. (1988). Synthesis of type II collagen is decreased in cartilage cultured with interleukin 1 while the rate of intracellular digestion remains unchanged. *Collagen. Rel. Res.* 8: 393-405.

120. van der Rest M, Rosenberg LC, Olsen BR, Poole AR. (1986). Chondrocalcin is identical with the C-propeptide of type II collagen. *Biochem. J.* 237: 923-5.

121. van Meurs J, van Lent P, Stoop R, Holthuysen A, Singer I et al. (1999). Cleavage of aggrecan at the ASN^{341}-PHE^{342} site coincides with the initiation of collagen damage in murine antigen-induced arthritis. A pivotal role for stromelysin 1 in matrix metalloproteinase activity. *Arthritis Rheum*. 42: 2074-84.

122. van Spil WE, Jansen NW, Bijlsma JW, Reijman M, Degroot J et al. (2012). Clusters within a wide spectrum of biochemical markers of osteoarthritis: data from CHECK, a large cohort of individuals with very early symptomatic osteoarthritis. *Osteoarthritis Cartilage*. 20: 745-54.

123. van Vulpen LF, van Meergeren ME, Roosendaal G, Jansen NW, van Laar JM et al. (2015). Biochemical markers of joint damage increase shortly after a joint bleed; an explorative human and canine in vivo study. *Osteoarthritis Cartilage*. 23: 63-9.

124. Verstappen SMM, Poole AR, Ionescu M, King LE, Abrahamowicz M et al. (2006). Radiographic joint damage in rheumatoid arthritis is associated with differences in cartilage turnover and can be predicted by serum biomarkers: an evaluation from 1 to 4 years after diagnosis. *Arthritis Res. Therapy*. 8: R31.

125. Vilar JM, Rubio M, Spinella G, Cuervo B, Sopena J, Cugat R et al. (2016) Serum collagen Type II cleavage epitope and serum hyaluronic acid as biomarkers for treatment monitoring of dogs with hip osteoarthritis. *PloS ONE*. 11: e0149472.

126. Wasilko SM, Tourville, TW, DeSarno MJ, Slauterbeck JR, Johnson RJ et al. (2016). Relationship between synovial fluid biomarkers of articular cartilage metabolism and the patient's perspective of outcome depends on the severity of articular cartilage damage following ACL trauma. *J. Orthop. Res*. 34:820–7.

127. Wei L, Fleming BC, Sun X, Teeple E, Wu W et al. (2010). Comparison of differential biomarkers of osteoarthritis with and without posttraumatic injury in the Hartley guinea pig model. *J. Orthop. Res*. 28:900-6.

128. Wu W, Billinghurst RC, Pidoux I, Antoniou J, Zukor D et al. (2002). Sites of collagenase cleavage and denaturation of type II collagen in aging and osteoarthritic articular cartilage and their relationship to the distribution of matrix metalloproteinase 1 and matrix metalloproteinase 13. *Arthritis Rheum*. 46: 2087-94.

129. Yamaguchi S, Aoyama T, Ito A, Nagai M, Iijima H et al. (2013). Effects of exercise level on biomarkers in a rat knee model of osteoarthritis. *J. Orthop. Res*. 31:1026-31.

130. Yoshida H, Kojima T, Kurokouchi K, Hanamura H et al. (2013). Relationship between pre-radiographic cartilage damage following anterior cruciate ligament injury and biomarkers of cartilage turnover in clinical practice: a cross-sectional observational study. *Osteoarthritis Cartilage*. 21:831-8.

131. Yoshimura M, Sakamoto K, Tsuruta A, Yamamoto T, Ishida K, Yamaguchi H and Nagaoka I (2009). Evaluation of the effect of glucosamine administration on biomarkers for cartilage and bone metabolism in soccer players. *Int. J. Mol. Med*. 24:487–94.