**IBEX collagen biomarker assays and their application: a brief review of recent literature (May, 2014)**

**A.Robin Poole, PhD, DSc**

**Professor Emeritus, McGill University**

**a.poole@mcgill.ca**

***Collagen turnover: Introduction to C2C, C1,2C and CPII immunoassays.***

Type II collagen is the predominant (by mass) collagen in hyaline cartilages including articular, whereas type I collagen is the major collagen found, for example, in joint synovial and capsular tissues and in tendon, ligament, bone and skin. These collagens are degraded mainly by collagenases as part of physiology and this degradation is more pronounced in pathology.

Biomarkers are available to detect collagenase-mediated cleavage generated neoepitopes of fibrillar collagens I and II in serum and synovial fluid. The C2C assay, previously called the Col2-3/4C long mono assay (1), is specific for the detection of cartilage type II collagen cleavage products in serum and synovial fluid. It has been used for urine in some cases although it was not designed for such analyses.

The assay for cleavage products of both types I and II collagens in serum and synovial fluid (C1,2C assay, previously called Col2-3/4 C short assay) can also be used in urine (2). The serum assay for the c-propeptide of type II procollagen (3, a measure of its synthesis) is called the CPII assay. It can also be used for synovial fluid.

Cleavage by collagenases is known to increase in arthritic articular cartilages in osteoarthritis (OA, 2) as well as in inflammatory arthritis such as rheumatoid arthritis (RA, 1), psoriatic arthritis (PsA) and ankylosing spondylitis (AS). In AS there is a primary involvement of spinal joint destruction where both types I and II collagens mainly constitute the nucleus (type II) and surrounding annulus (types I and II) of the intervertebral discs.

Initially, a competitive inhibition serum assay for C2C was created (C2C, 1) and used to study type II collagen cleavage. Very recently a new C2C sandwich assay for urine was developed that detects a 45 mer peptide that is markedly increased in urines of OA patients (4). This new assay (IB-C2C-HUSA™) detects little or no reactivity in serum but is capable of much better detection of the increase in type II collagen cleavage observed in urine with onset of early degeneration of cartilage damage and OA (see below).

With onset of increased cleavage in articular cartilages of type II collagen, such as in early OA, there is an accompanying increased synthesis of this collagen by

chondrocytes. This can be measured by the release of the c-propeptide of type II procollagen using a competitive inhibition assay (3). But in OA this newly synthesized collagen is quickly cleaved (5). Type II collagen cleavage and synthesis is a normally in balance in healthy cartilage. In OA an increased emphasis on degradation (C2C) over synthesis (CPII), depicted by the C2C : CPII or C1,2C : CPII ratios, is a reflection of arthritic changes favouring cartilage degradation and loss. (6). Recent studies have indicated that in both normal and OA cartilages degradation of the newly synthesized collagen is what is mainly detected by the sC2C assay (ARPoole, J Cibere et al, submitted).

These assays were designed for use in human studies. But there are examples of their application to analyses of joint fluids, sera and urines in experimental studies in animals. Examples of these and human usage are also described below.

***Human studies of osteoarthritis using competitive inhibition serum assays***

Using MRI, positive correlations were observed between the C2C serum assay and male OA knee T2 images (7). MRI and radiographic analysies 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 (8). The risk of pre-POA versus no OA increased with increasing urine levels of C2C and C1,2C. However, the ratios of urine measures of sC2C or sC1,2C to sCPII were better than individual biomarkers at differentiating the subgroups. Serum analyses of these degradation biomarkers failed to distinguish between the different groups. Also, there were no correlations between serum and urine measurements with the sC2C and sC1,2C assays. This pointed to the fact that the cleavage neoepitope-containing fragments were different in serum and urine.

Others have also found that early radiographic onset of knee OA is accompanied by increases in serum C2C and C2C:CPII and a decrease in CPII (9). In those patients with no radiographic OA changes but with knee pain C2C and CPII were increased pointing to early changes in cartilage turnover that are characteristic of degeneration.

When knee or hip OA patients were haplotyped , the C2C, CPII and the C2C: CPII ratio were significantly increased in sera of OA patients carrying the haplogroup H compared to OA carriers of the J haplogroup (10). Recent studies of sera of knee OA patients revealed that the serum C2C assay levels were increased in these patients compared to controls (11). Improved identification of early OA should be seen in future with combined use of such biomarkers and haplotyping.

Patients with hip OA also exhibit differences in serum levels of C2C and CPII according to the group analyzed, namely only hip OA, multiple site OA and unilateral

hip OA (12). The latter group exhibited a significant inverse correlation between minimum joint space width and serum C2C.

Treatment studies in patients with knee OA receiving oral salmon calcitonin revealed a significant reduction in urine C2C, not seen in the placebo group, that was accompanied by a significant improvement in joint function (13). MMP-13 levels were also decreased, a collagenase thought to be mainly responsible for generation of the C2C neoepitope (5).

In investigations of the progression of knee OA, the ratios of C2C: CPII and C1,2C: CPII both show an almost significant relationship to radiographic assessment of disease progression not seen with the individual biomarkers (14). This again points to the importance of measuring the balance between type II collagen degradation and synthesis as we shall see below when using the new IB-C2C-HUSA™ urine assay.

A review of serological biomarkers of erosive and non-erosive hand osteoarthritis Ramonda et al concluded that C1,2C (Col2-3/4 C short) was one of the most useful and most discriminatory biomarkers for the study of hand OA (15).

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 (16).

Patients at risk for OA following knee ACL injury with or without abnormal joint space width (JSW, to detect loss of or damage to articular cartilage) showed an increased ratio of urine C1,2C: serum CPII compared to controls at 1 and 4 year follow-up (17). Those with abnormal JSW had increased uCTX-II: serum CPII compared to controls. The latter may reflect early osteophyte formation in view of the main origin of this biomarker in calcified cartilages (18) and association with the turnover of calcified tissues (19).

In another study following knee ACL rupture, in which baseline serum pre-injury samples were available, subsequent serum changes in C2C, C1,2C, CPII and CS846 (the aggrecan synthesis biomarker) were observed compared to age-related changes in uninjured controls: these indicated alterations in cartilage matrix turnover induced by the injury (20). The same group has importantly discovered that patients at much increased risk for ACL rupture (ORs range 10-20 fold according to the biomarker) can be identified prior to injury by pre-injury differences in serum C2C, C1,2C and CPII (S. Svoboda, K. Cameron et al, submitted). This suggests fundamental genetic and/or gait related differences in cartilage metabolism, measurement of which offers new ways of potentially avoiding sports and other causes of knee injuries.

Recommendations on the use of molecular biomarkers in the development of drugs intended for the treatment of OA that have been submitted to the FDA include the

IBEX biomarkers C2C, C1,2C, CPII and CS846 (21). These and other recommended biomarkers, with the addition of the new IB-C2C-HUSA™ assay, are now the subject of further study of their ability to identify disease onset and progression as part of the NIH public/private OA initiative involving the Foundation for NIH and industry.

### *Animal studies of experimental and natural osteoarthritis using competitive inhibition assays*

Although these assays were developed for human studies based upon human neoepitope sequences and proteins, close similarities and identities in sequences across species has enabled their use in a number of animal biomarker studies as a result of species cross-reactivity of antibodies. Some of these are described here.

In equine studies of osteochondral injuries, synovial fluid (SF) analyses with sC2C revealed increases in injured joints that correlated with the severity of injury (22). In early OA in horses, synovial fluid (SF) levels of C1,2C, CPII and the IBEX assay for aggrecan synthesis CS 846 were all increased (23). In sera these same biomarkers were also elevated in OA compared to exercised horses without OA. (23).

Induction of unilateral OA in dogs is accompanied by elevations in C2C in SF starting at 3 weeks followed by serum C2C at 12 weeks (24). Again, serum CS846 was also elevated at 3 and 12 weeks. In natural onset of OA in dogs, SF C2C is also elevated compared to unaffected dogs (25).

Murine osteoarthritis has also been studied with these biomarkers revealing elevations of serum C2C (26). In guinea pigs, onset of natural OA in the Hartley strain is accompanied at 4 months of age by an early elevation of the ratio of serum levels of C2C: CPII compared to strain 13 which is OA resistant (27). Elevations of SF C2C, as well as MMP-13 that can generate the C2C neoepitope, also occur in Hartleys and even greater increases in the Hartley strain that underwent ACL section to produce more severe OA (28).

### *Human studies of erosive inflammatory arthritis (IA) using the competitive inhibition assays*

Unlike most cases of OA, patients with IA, such as rheumatoid arthritis (RA), usually involves multiple joints and therefore the contribution of pathological extracellular cartilage collagen turnover is likely much greater to the circulating pool of degradation products.This provides more discrimination of disease presence and activity. Initial analyses of sera from patients with RA revealed increases in the C2C neoepitope (1). Subsequent serum analyses showed that compared to RA patients with slow radiographic changes, those with rapid radiographic progression (aggressive disease) had persistently elevated levels in sera of C1,2C, C2C and CS846 but not CPII over a 4 year period. The values after one year predicted subsequent progression, especially in the case of C2C (29).

In a biologic treatment study of patients with IA, examination of the balance between type II collagen (C2C) and type I collagen (C1,2C) degradation products and synthesis of type II collagen (CPII) has revealed that after 1 month of treatment changes in these three biomarkers predicted radiographic outcome in 88% of patients after 1 year (30). In a biologic treatment study with infliximab, serum levels of the ratio C2C : CPII were decreased on treatment, compared to baseline, in early RA regardless of the EULAR response grade (31). The change in C2C: CPII over 54 weeks correlated with the change in DAS28 levels, radiographic progression and patient function (HAQ). But C2C: CPII remained unchanged in established RA.

Studies of other types of IA have also shown differences in these collagen biomarkers. In psoriatic arthritis, increased levels of CPII : C2C were observed compared to those with psoriasis without joint disease (32). Patients with ankylosing spondylitis (AS) treated with etanercept for 16 weeks revealed a reduction in serum C2C and an increase in serum CS 846 (33). In another similar study patients treated with etanercept also exhibited a decrease in C2C after 12 months: after 24 months CPII was increased (34). Both studies point to less cartilage damage (reduced C2C) and onset of reparative responses reflected by increases in CS846 and CPII.

The value of serum measurements in RA and other inflammatory diseases, copared to their more limited value in OA, is most likely due to the greater number of diseased joints involved that contribute to the serum pool.

***Animal studies of experimental IA***

In a rat model of IA using streptococcal cell wall induced polyarthritis, serum C2C increases with onset of acute and more so in chronic disease. These increases are much reduced with treatment of the arthritis (35). Rabbits with monoarticular IA also show an increase in C2C in joint fluids of the affected stifle joint, but not in serum (36), probably because the change in the rabbit is monoarticular.

***Development of a new urine IB-C2C-HUSA™ sandwich assay by IBEX that is proving to be of much greater value in OA than the serum assay.***

With availability of Mass-Spec information detailing the structure of a type II collagen C2C neoepitope-containing peptide that is increased in urines of OA patients (4), a new sandwich assay has been developed by IBEX to specifically measure in urine the OA pathology-related 45 mer peptide containing the C2C neoepitope.

Initial studies with this assay reveal that it detects the generation of a pathology-related cartilage collagen peptide in urine although little or no reactivity is seen in serum. This urinary peptide is progressively increased with onset and progression of cartilage degeneration in a population-based cohort examined radiologically and

by MRI. Importantly, in this cohort it can discriminate between the normal knee, pre-radiologic articular cartilage degeneration and radiologic OA in a superior manner to that seen with the C2C competitive inhibition assay applied to the same urine samples. Moreover, the new assay alone is also more predictive of the progression of cartilage degeneration compared to the C2C assay for urine (AR Poole, J Cibere et al, submitted). In this same study there was no correlation between the serum C2C assay and the IB-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 with C2C in the same patient (8).

This new assay has also been used to examine urine values in patients where knee cartilage lesions were observed. In patients with Outerbridge grade II or higher clear increases were detected (37). The new urine assay produces similar results to those obtained earlier by analyses with C2C of synovial fluids in patients with pre-radiographic cartilage degeneration characterized by Outerbridge grade II and higher grade lesions; here the number of lesions correlated directly with the C2C level (38). In the study by Tamm et al (37), there was also a positive correlation of IB-C2C-HUSA™ results and joint symptoms as well as joint function. These correlations were strongest when IB-C2C-HUSA™ was expressed per creatinine.

We await application of this new urine assay to patients with RA and others with inflammatory arthritis

***References***


1. 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 Immunological Methods, 294:145-53.
2. 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.
3. 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.
4. 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.
5. Dahlberg L, Billinghurst C, Manner P, Ionescu M, Reiner A et al. (2000). Collagenase-mediated cleavage of type II collagen is selectively enhanced in osteoarthritis cartilage and can be arrested with a synthetic inhibitor which spares collagenase-1 (MMP-1). Arthritis Rheum 43: 673-82.
6. 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.

7. 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.
8. 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 osteoarthritisin: a population-based cohort. Arthritis Rheum 60: 1372-80.
9. 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
10. Fernandez-Moreno M, Soto-Hermida A, Oreiro N, Pertyega S, Fenandez-Lopez C et al. (2012). Mitochondrial haplogroups define two phenotypes of osteoarthritis. Front. Physiol. 3:129.
11. Lin H, Wu X. (2014). Detection of urine C2C and trace element level in patients with knee osteoarthritis. Cell Biochem Biophys. E-pub.
12. 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.
13. 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.
14. 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.
15. Ramonda R, Lorenzin M, Modesti V, Campana C, Ortolan A, et al. (2013). Serological markers of erosive hand osteoarthritis. Eur. J. Intern. Med. 24(1):11-15.
16. 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.
17. Tourville TW, Johnson RJ, Slauterbeck JR, Naud S, Beynnon BD. (20!3). 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.
18. 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.
19. 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.
20. 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.
21. 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.
22. Trumble TN, Scarborough AB, Brown MP. (2009). Osteochondral injury increases type II collagen degradation products (C2C) in synovial fluid of Thoroughbred racehorses. Osteoarthritis Cartilage 17:371-74.
23. 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: 1196-2014.
24. 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.
25. 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.
26. 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.
27. Huebner JL, Kraus VB. ( 2006), Assessment of the utility of biomarkers of osteoarthritis in the guinea pig. Osteoarthritis Cartilage. 14: 923-30.
28. 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.
29. 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 Ther 8: R31.
30. 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.
31. 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
32. 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.

33. Maksymowych WP, Poole AR, Hiebert L, Webb A, Ionescu M et al. (2005). Etanercept excerts beneficial effects on articular cartilage biomarkers of degradation and turnover in patients with ankylosing spondylitis. J Rheumatol 32: 1911-17.
34. 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.
35. 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.
36. 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: 120-27.
37. 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.
38. Yoshida H, Kojima T, Kurokouchi K, Takahashi S, 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: 831-38.