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Biochemical Markers as Surrogate Endpoints of Joint Disease

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This chapter will discuss the potential for biochemical markers as surrogate endpoints for clinical outcome in drug trials and management of joint diseases. The focus of the chapter is on osteoarthritis (OA), but the basic aspects of biomarker development and validation, and their qualification as surrogate outcomes apply to both OA and other joint diseases such as rheumatoid arthritis (RA).

Osteoarthritis: the disease and the needs

OA is most common in the hands, knees, hips and spine. A single joint may be involved, but most individuals have several affected joints at different stages of disease development. OA is steeply age-related. Most people over the age of 70 have some radiological evidence of OA in some of their joints. As our populations age, degenerative skeletal disorders impose an increasing burden in health care costs and lost life quality. Today there are 600 million people over 60 on the planet. This will double by 2025 and double again by 2050 according to the World Health Organization (WHO).

OA is by far the most common type of arthritis and is a leading cause of chronic disability (Kaplan & Laing 2004). Disease burden due to OA is on the top 10 list based on the DALY score of all chronic and acute conditions¹. For women in Europe the proportion of total disease burden is greater than 6% and for men 3% of total DALYs. Although OA is often regarded as a disease of the elderly, it is of note that the peak of OA disease burden occurs around age 60 (variable in different world regions), and is very significant already in the age group of 30-44 (1).

A recent report from the WHO characterized two conditions as “high burden diseases with no curative treatments”: OA and Alzheimer’s disease (1). The report further stated that currently available treatment is inadequate and that “Both are common and increasing among the elderly, and available treatment is ineffective in reversing disease progression. A major challenge for both diseases is the absence of biomarkers which could be used to diagnose and monitor the progression of disease or the effect of treatment. Continued support is needed for basic research on these diseases.

¹ DALY (Disease Adjusted Life Year) is an integrated measure of mortality and disability, combining mortality and morbidity in a single measure. One DALY can be thought of as one lost year of ‘healthy’ life, and the burden of disease as a measurement of the gap between current health status and an ideal situation where everyone lives into old age free of disease and disability.

Pharmaceutical companies invest heavily in research on both of these diseases but there are major biological challenges in understanding and then reversing these progressive diseases.”

Treatments for OA are available that mitigate pain and improve function, but there are at present none that can cure, reverse or halt OA disease progression (disease modification). New compounds are under development in the pharmaceutical industry for treatment of OA related symptoms and for disease modification. However, considerable challenges exist in identifying and selecting the most promising treatment targets and in monitoring the efficacy of a particular compound against a target in early stage drug development. This represents an important area of use for future OA biomarkers. Biomarkers may have further public health benefits in that they may make it possible to reduce the numbers of patients needed in early stage drug development, speeding drug development and testing and reducing the number of persons exposed to new compounds where understanding side effects is yet limited. As with treatment of any other chronic disease, long-term safety issues are a major concern and may require large scale clinical studies to assess in the relevant populations. Surgical treatment of advanced OA with severe symptoms is an effective treatment, but is only relevant for a minority of all patients with OA.

OA develops as focal areas of damage to the articular cartilage, typically in the load-bearing areas, associated with new bone formation at the joint margins (osteophytes), changes in menisci, ligaments and in the subchondral bone, variable degrees of usually mild synovitis, and thickening of the joint capsule. When these pathological features are advanced they are recognizable on plain radiographs as joint space narrowing, osteophytes, and sometimes changes in the subchondral bone. Magnetic resonance imaging (MRI) of joints is a rapidly developing technique with the potential to identify and monitor these features of joint pathology at much earlier stages than plain radiographs (See Chapters 14 and 15).

The patient-relevant problems associated with these pathological and radiographic changes include all or some of the following: joint pain in use and sometimes at rest, short lasting inactivity stiffness of joints, restricted range of movement, and instability and lack of confidence in joint function. Pain is particularly important, and OA may be

the most significant cause of the great prevalence of regional joint pain in older people. However, the correlation between radiographic evidence (whether by X-ray or by MRI) of OA and the symptomatic disease is rather weak. This raises issues relating to the definition of the 'OA disease' and the extent to which our efforts should be directed towards the treatment of the pathology of joint damage, or the causes of pain and physical disability (2).

OA is a multifactorial disease involving endogenous factors, such as age, sex, and genes, as well as exogenous environmental factors such as joint load and trauma. Convergence of these risk factors leads to the initiation of the disease state (Figure 1). Whether the factors that lead to disease initiation are the same that drive the disease from its initiation to any of its various outcomes, such as osteophyte formation, cartilage loss, inflammation, or pain, is unclear. To monitor OA incidence, a group of at-risk individuals are followed along a continuum progressing from a more or less normal state to a disease state. At some point, the individual crosses a borderline between what is recognized as absence of OA to presence of OA. The distinction between incident cases of OA and progression of prevalent cases depends on where along the continuum patients are considered to have overt OA.

Publications on the natural history of OA remain sparse, but OA is considered a progressive condition. While this view may be correct on the group and population level, it may be less so on the level of the individual. Several recent studies suggest that the group mean rate of progression of joint damage reported in earlier studies is affected by a small number of fast progressing individuals. These observations are accompanied by other reports that describe a similar heterogeneity in the progression of OA symptoms in a susceptible population. It also appears likely that even when there is disease progression, it is intermittent. We may thus ask whether we can identify early on either those patients who will experience rapid progression of disease or, equally important, those patients who will not. Clearly, patients at risk for rapid progression should be at the focus of our current and future efforts to slow or stop disease progression in OA. These patients also, in consequence, serve as a population of interest for clinical trials of new disease-modifying drugs for OA, while a natural history study should be representative of the whole population (3) (Figure 2).

Studies of OA indicate that obesity, number of joints affected, ligament and meniscus integrity, and genetics are associated with progression of structural joint changes. Limb malalignment, both static and dynamic, is another potent risk factor for progression of structural change in knee OA. Evaluations of the relationship between pain severity and OA disease progression (structure and or symptoms) have produced variable results. Other factors reported to be linked to an increased risk of progression of radiographic signs of OA and loss of joint cartilage include low vitamin D levels, low vitamin C intake, serum testosterone in men, and low bone density.

Elements making up a high risk profile would thus likely include demographics, signs and symptoms, structural changes, and family history (genetic background). Some recent results suggest that OA biomarkers may now begin to contribute to the risk profile, see further discussion below.

Development of a high risk patient profile is still complex, however, since we do not yet fully understand the interactions between different risk factors for OA. For example, if we assume a “background” rate of progression (or incidence) of OA in the population, what is the effect of adding a specific risk factor? Does the proportion of individuals entering the ‘OA pathway’ increase? Does the rate of disease progression remain the same or does it change in the presence of the added risk factor? Do certain risk factors for OA become active only in certain environments and in the presence of other factors, within a “permissive environment”? Large-scale investigations such as the Osteoarthritis Initiative are underway to generate such information (4). OA may thus serve as a ‘case study’ of a high burden disease lacking curative treatment, and where biomarkers would have significant utility.

Clinical assessment and biomarkers – definitions and classifications

There are several ways by which OA outcomes can be assessed: (A) patient-related measures of joint pain, impairment and disability [scores such as WOMAC (5) or KOOS (6), and others (7)]; (B) measurements of the structural (anatomical) changes in the affected joints [plain radiographs (8), magnetic resonance imaging (9), arthroscopy (10), high frequency ultrasound (11)]; or (C) measurements of the disease process exemplified by changes in metabolism or functional properties of the articular cartilage,

subchondral bone or other joint tissues [biochemical markers of cartilage and bone metabolism, bone scintigraphy, measurement of cartilage compression resistance].

These different dimensions of outcome are related to the concept of defining an endpoint for use in measuring OA disease development or for use in a clinical trial when comparing two different treatments. In the greater context of treatment of a medical condition such as OA, how a patient feels, functions or survives is the most relevant outcome. Other measures and endpoints may be relevant as well, but need to be validated against this gold standard for their long-term value to be established, and be classified as surrogate outcome measures.

As exemplified in the previous section of this chapter, there is a recognized need for biomarkers in OA. Examples of potential uses include but are not limited to: exploring disease mechanisms and dynamics, identifying molecular targets for treatment, identifying patients-at-risk for rapid disease progression, monitoring effects of disease-monitoring therapy and predicting clinical response, and to tailor treatment to response. The need for biomarkers is particularly acute in the proof-of-concept stages of drug development of disease-modifying OA therapy. It may further be speculated that access to useful biomarkers in OA could have public health benefits in that the number of patients that would need to be exposed to a new drug in development might be decreased. Biomarkers could help speed drug development, and allow testing of more alternatives in this complex disease area. Further rationale for continued OA biomarker research is provided by the increasing awareness of the severe limitations of plain radiography as a method to monitor OA outcome.

A *biomarker* may be defined as a structural or physical measure, or a cellular, molecular or genetic change or feature by which alterations in a biologic process can be identified and monitored. A biomarker may thereby have diagnostic or prognostic utility.

Biomarkers, in order to be useful, must be reliably and reproducibly measured by standardized, published methods in several laboratories, and validated to prove that they are indeed measuring the intended analyte and/or process with sufficient specificity.

A *surrogate marker or endpoint*, on the other hand, is a measurement or biomarker that can serve as a substitute for a clinically meaningful outcome or endpoint. A surrogate marker might thus also serve to predict the effect of a clinical intervention.

A *clinical endpoint* may be defined as a characteristic or variable that measures how a *patient* feels, functions or survives. It follows from these definitions that even in the best of cases, only some biomarkers of OA may serve as surrogate endpoints for OA. In order for the biomarker to be validated as a surrogate endpoint, it must be shown that its measurement can serve as a reliable substitute for, or predict, a clinically meaningful endpoint (12, 13).

A significant challenge in the validation of a surrogate marker is that its measurement may not take into account adverse events, since the processes associated with an adverse event may not be monitored by the marker. Such adverse events may null all or some of the treatment benefit, and require identification of biomarkers specific for such events. Further, a surrogate marker may not register all beneficial effects of treatment if these are not in the marker pathway. Although a biomarker may have good face validity as a surrogate outcome, changes in its measurement may not monitor the intended molecular or cellular process in the tissue it is thought to, leading to erroneous conclusions.

Biomarkers may have several different potential uses, and a general classification has been proposed (14). According to this framework, a natural history marker is defined as a marker of disease severity that reflects underlying pathogenic mechanisms and predicts clinical outcome independent of treatment. Such biomarkers, *type 0*, are identified as prognostic in longitudinal history studies of the disease. Type 0 markers can be used for baseline stratification in clinical trials, and as milestones of disease progression in the natural history of the disease. A next suggested stage in marker development is to assess the influence of treatment on a promising prognostic type 0 marker. Such a *type I* biological activity marker is defined as one that responds to therapy. A type I biomarker would likely be evaluated in early stage clinical trials with the aim of providing proof-of-concept that a new treatment has promising activity related to its suggested mode of action. Possibly, a type I biomarker could be used to help estimate optimal drug dosing. Finally, a *type II* biomarker (or a composite of several markers) may be defined as one that predicts a subsequent favorable clinical outcome, and thereby accounts for the clinical efficacy of an agent or treatment. Such a biomarker would be defined as a surrogate marker of therapeutic efficacy. It is more likely, however, that any surrogate marker will explain only a part of the clinical efficacy, the proportion of treatment effect explained (PTE) (15). As discussed (14), a

correct interpretation of the PTE requires a thorough understanding of the underlying mechanisms of disease and drug activity. Only if it is known that the agent operates primarily through its action on the marker and the marker is directly in the causal pathway of the disease can marker results be interpreted reliably. Conclusive proof of this remains a challenge for many currently explored biochemical markers of OA.

Validation of a biomarker for its intended use (type 0, I or II) should follow a stepwise approach, beginning with an initial hypothesis on pathogenesis. Early studies are usually descriptive and cross-sectional cohort studies of limited size. Subsequent validation stages need to expand significantly in size and be longitudinal, initially retrospective and later prospective. For biomarkers of type I or type II, access to an active intervention is required. The continued absence of a drug or treatment with an unambiguous disease-modifying activity in OA (however defined, see above) makes any attempt to validate a type II biomarker for OA problematic at this time. Current biomarker work in OA is therefore largely limited to a search for type 0 and type I biomarkers.

For a disease-modifying therapy in OA, it may be argued that a clinically meaningful outcome should combine evidence of joint structure (or joint survival) benefit with more direct patient-relevant benefit relating to pain, function or joint-related quality-of-life. This clinical outcome would then serve as the gold standard against which any biomarker aspiring to be defined as a surrogate OA marker (type II) needs to be validated. Investigators in the field need to agree on a standard clinical endpoint to be used for each proposed use of a biomarker or surrogate marker. If a biochemical marker is validated against 'structural' joint outcome only, it will serve as a case of one biomarker being related to another, and not against a clinical outcome. However, this does not necessarily mean that a biomarker not fully validated as a surrogate outcome is not useful. It may indeed be so, in that it may be able to support the identification of a treatment target, to monitor in vivo or in vitro a specific cellular or molecular process of interest in drug development, etc. Biomarker validation is not all or none, but a process of gradual strengthening of evidence. In validating an OA biomarker, studies will need to account for interactions generated by which joint(s) are studied, disease stage, co-morbidities and medications, ethnicity, sex, age, body mass, and yet other factors.

The status of OA biomarkers: strengths and limitations

OA and other joint diseases such as RA are associated with a loss of the normal balance between synthesis and degradation of the structural components of the extracellular matrix. These components are necessary to provide articular cartilage, menisci, ligaments and bone with their normal biomechanical and functional properties. Concomitantly, synovitis develops which is usually much less pronounced in OA than in RA. These processes result in the destruction of joint cartilage, menisci and ligaments, with extensive remodeling of subchondral bone. The active processes in the joint, involving changes in both synthesis and degradation, result in the altered release of matrix molecules, proteolytic molecular fragments, and other molecules involved in their altered metabolism such as proteases, cytokines, chemokines, growth factors, etc. For the synovial joint, the joint fluid is a likely first and most proximal compartment where these potential biomarkers, intact or fragmented, may be present. Products released into the synovial compartment may be removed from there by capillary and lymphatic flow to appear in the blood circulation, and in some cases they may survive metabolism and appear in the urine after further processing by the kidneys.

The quantitative relationship between biomarker and tissue turnover – Although simple in principle, the relationship between changes in biomarker concentrations in a body fluid compartment and changes in joint tissue metabolism is complex and not fully understood (Figure 3). To use joint cartilage as an example: the concentration of a marker of cartilage matrix degradation in joint fluid will depend not only on the rate of degradation of hyaline joint cartilage matrix, but also on the clearance rate of the molecule or fragment in question from the joint fluid compartment (16), and the amount of cartilage matrix remaining in the joint (17). Since the clearance of macromolecules from the joint fluid compartment to the lymphatics or directly to capillaries may be increased by inflammation (16), differences in the rates of release of markers from joint cartilage into joint fluid between control joints and diseased joints with inflammation may actually be underestimated. The contribution of biomarker release into systemic circulation from other joints with OA or from normal joints adds complexity, as may contributions from other hyaline cartilage structures in the body. For example, articular

cartilage makes up only 8% of the total hyaline cartilage of a young dog, so this is not a trivial hurdle (18). In monoarticular disease, any markers released from the affected joint are thus mixed with markers released from normal cartilages. Hence, determinations of cartilage markers in serum or urine may be of more use in polyarticular or systemic disease, and may be less likely to be useful in monoarticular disease where measurement of joint fluid may provide a more accurate insight into the local pathology.

Quantitatively, type II collagen is the most abundant component of joint cartilage and its destruction is a central, irreversible feature of joint failure, so it presents an attractive target as a biomarker. However, in skeletally mature adults, type II collagen is found in articular cartilages, fibrocartilages (intervertebral discs, menisci, etc.), respiratory tract cartilages, rib cartilages and the insertion sites of tendons and ligaments into bone. Small amounts also occur concentrated in tissues of the inner ear and the eye. The source of any collagen II fragments found in body fluids therefore cannot be assumed to be primarily from joint cartilages without any further data on the source and a likely mechanism of generation. In relation to type I collagen and in the body as a whole, collagen type II probably represents in the order of 1%. On the other hand, the turnover rate of type II collagen in adult cartilage will normally be very low compared with bone type I collagen, for example, so elevated degradation and synthesis in even a single joint might be expected to raise systemic levels of fragments significantly.

Reported estimates from MRI studies of the volume loss of articular cartilage in knee OA are in the range of 200-500 cubic mm per joint per year (19). If representative, this corresponds to 40-100 mg of type II collagen per year or about 0.1 to 0.3 mg (0.3 to 1 nmol) per day. Such levels are in a range that a sensitive immunoassay can quantify if diluted into the blood or excreted into urine.

Inflammation, OA and biomarkers – Hyaluronan concentrations are high in joint fluid, and hyaluronan is synthesized by cells of the synovium as well as by cells of other connective tissues. Increased hyaluronan concentrations in serum correlated with OA joint space width and disease progression in some studies, but not in others (20-23). Synovitis in OA has a significant effect on serum COMP, suggesting that COMP levels may relate to joint inflammation in OA (24). Both chondrocytes and synovial cells

produce MMP-3 (stromelysin-1), but the cell number and rates of synthesis in synovial tissue may be higher in the inflamed synovium than in cartilage, so that a significant proportion of the MMP-3 detected in joint fluid, plasma or serum originates in the synovium. High concentrations of MMP-1, MMP-3 and TIMP-1 protein are found in joint fluid shortly after injury, perhaps as a reflection of synovial activation and inflammation (25-26). Interestingly, plasma levels of MMP-3 were shown to be predictive of knee joint space narrowing over 16 months in a prospective, longitudinal OA clinical trial (27). Reports on a possible relationship between serum C-reactive protein (CRP) and OA have been variable, with some suggesting a relationship and others failing to find one (28-31). Several studies suggest that serum CRP, like several other OA biomarkers, is influenced by obesity, body mass index and co-morbidities, which is likely to confound interpretation of results.

The results of these studies, using a variety of biomarkers, suggest that inflammation is a feature of at least some phases of OA. This view is consistent with recent reports showing increased levels in synovial fluid and serum of OA patients of several different cytokines and chemokines (32-33). It is likely that in some phases of the OA disease process inflammation may be a relevant pathogenic driver and relevant treatment target in OA (34-36).

Neopeptides add specificity – Even if specific for the particular structure (epitope) detected, as well as for its target molecule, many of the currently used biomarker assays lack specificity for the metabolic process generating them. In contrast, immunoassays that rely on the detection of a neopeptide generated through specific proteolytic substrate cleavage can provide information on the activity of that specific proteolytic pathway (37). Such information is particularly valuable for the monitoring of disease modifying therapy of OA and any other joint diseases for which proteolytic inhibitors are now being developed. Type II collagen and aggrecan may serve as examples of the role of neopeptide-specific biomarker assays.

Lessons from studies of degradation of collagen in bone and cartilage – The evolution of collagen degradation products as biomarkers of bone resorption in the osteoporosis field may be informative for OA biomarker research. Pyridinoline cross-links (HP and LP) and peptides containing them are present in blood and urine from tissue collagen

degradation (Figure 4). Since there is no mechanism for their metabolism, these residues give an index of collagen breakdown. Urinary pyridinolines (HP and particularly LP) measured by HPLC give a more accurate index than hydroxyproline, a traditional marker of systemic bone resorption.

Even more specific are assays for telopeptide fragments containing the cross-linking residues (38-39). The cross-linked telopeptides of bone collagen (N-telopeptide-to-helix - NTx, and C-telopeptide-to-helix - CTx (Figure 5); survive into blood and urine and can be isolated from urine as a discrete low molecular weight peptide pool (<2kDa) (38, 40-41). Antibodies raised that recognize them as proteolytic neoepitopes have formed the basis of immunoassays developed to quantify NTx and CTx fragments as biomarkers of bone resorption. The NTx epitope was shown to be generated during osteoclastic degradation of bone collagen through the action of cathepsin K (42-43) and so is a marker of the process of degradation as well as specific to type I collagen (Figure 5).

Although bone collagen is the principal source of the pyridinoline cross-links in urine, other tissues also contribute, notably cartilage type II collagen in growing children (44). A C-telopeptide fragment containing HP from type II collagen was identified (41, 45). From the sequence of a collagen type II C-telopeptide fragment identified in human urine (45), a monoclonal antibody, 2B4, was prepared that recognizes the cross-linked structure (46) (Figure 5). An ELISA assay based on this antibody was able to monitor culture medium for collagen II breakdown products from IL-1-stimulated cartilage explants in vitro (47), increased urinary excretion in OA patients (48) and higher levels in knee synovial fluid of patients after joint injury (49), in dogs after ACL section (50) and in rabbits after meniscectomy (51). The same 2B4 assay applied to urine was also used to compare cartilage collagen degradation with bone resorption (NTx assay) in high-performance college athletes in three different sports: crew (rowing), cross-country and swimming (O'Kane, Eyre et al. unpublished). Interestingly, bone resorption was highest in the crew and lowest in the swimmers whereas cartilage degradation was highest in the runners and lowest in the swimmers. Each group showed statistically significant differences in each marker from the other.

From our unpublished results we know there isn't a simple correlation between urine, serum and synovial fluid in collagen II CTx epitope levels. As with the bone telopeptide markers NTx and CTx (see below), there is a need to determine the origin and fate of the proteolytic epitope (2B4) in the body fluid compartments. We suspect that in synovial fluid, large molecular fragments of type II collagen from articular cartilage are the source of the immunoassay signal, whereas in urine the peptides carrying the signal are small, and less than 2 kDa in size. The antibody will detect the neoepitope, whether on the end of the whole molecule or at the end of a short peptide. The peptides in urine probably originate mostly from osteoclast-degraded mineralized type II collagen as do type I collagen NTx and CTx (41). We suggest that larger cross-linked fragments that originate from matrix metalloproteinase-driven mechanisms are degraded to free pyridinolines in the liver. This concept is supported compellingly in the recessive skeletal disorder pycnodysostosis caused by a homozygous null cathepsin K gene. Type I NTx, type I CTx (52) and type II CTx epitope levels are very low in urine in pycnodysostosis compared with age-matched normals, yet total urinary HP and LP are normal (unpublished). This can be explained if HP- and LP-containing products of collagen degradation are fully degraded to the free cross-links, which are then excreted (Figure 4). Without cathepsin K, resorbing osteoclasts demineralize bone but can't degrade the collagen, which is removed by macrophages or other phagocytic cells (53). Based on this information, we suggest that type II CTx fragments in urine may reflect primarily the breakdown and remodeling by osteoclasts of mineralized cartilage collagen (50).

Urine levels of type II collagen CTx fragments have been reported to correlate with arthritis severity, joint disease load and to predict OA progression (54). However, although this may fulfill the requirements for a type 0 biomarker, the results discussed above suggest that CTxII may not necessarily be a suitable type I biomarker showing therapeutic response related to a potential treatment effect on hyaline joint cartilage. CTxII levels in urine were shown to be suppressed markedly by the bisphosphonate risedronate in a phase III trial of this compound for slowing the progression of knee osteoarthritis (55). Though the marker had good face validity (assessing collagen type II destruction) and responded as anticipated for chondroprotection with marked suppression, the trial end-point of radiographic assessment of joint-space narrowing or

symptoms showed no risedronate benefit over the placebo-controlled arm. From animal studies we already knew that bisphosphonates suppressed the CTxII analyte in growing guinea pigs (56), and presumed this was a result of the inhibition of osteoclastic resorption of mineralized cartilage by active growth plates. Based on the predicted route of small cross-linked telopeptides to urine from osteoclast activity it seems likely that the main source of urinary CTxII is type II collagen in mineralized tissue, perhaps from joint remodeling that involves osteophytes and the tidemark cartilage interface with mineralized cartilage and bone, but also perhaps from skeletal sources other than joints. This illustrates the complexities and pitfalls inherent in using a type II biomarker as a type I biomarker without a thorough evaluation and understanding of the underlying metabolism.

Biomarkers of aggrecan degradation and turnover – Aggrecan degradation and loss has significant consequences for the resilience of joint cartilage, and experiments with cartilage explants even suggest that aggrecan loss is a prerequisite for collagen loss (57). While several assays have been developed to monitor aggrecan fragment levels in body fluids, they lack the specificity to detect activity of specific proteolytic cleavages of this molecule (58-60). Several lines of evidence indicate that aggrecanases, primarily ADAMTS-5, play an important role in aggrecan degradation in human joint disease (61-65). However, a role for matrix metalloproteases or other proteases in some phases of aggrecan turnover in the human joint is difficult to exclude (66), and there is little or no information on possible variations in proteolytic activity associated with the individual, with disease stage or with specific joint disease (Figure 6). For these reasons, assays that detect only aggrecan fragments resulting from specific protease activity would be helpful in understanding the relative roles of different proteases, and for monitoring efficacy in early compound screening and clinical development.

Other matrix components of joint cartilage – Cartilage oligomeric matrix protein (COMP) is an oligomeric pentameric glycoprotein present in cartilage, tendon, meniscus, ligament and some other connective tissues and may serve as yet another example of a matrix molecule released into body fluids in joint disease. Fragments of this molecule have been detected in synovial fluid and serum in OA and other joint diseases. The enzyme(s) responsible for COMP degradation in vivo have yet to be identified, and the fragment population present in body fluids is heterogeneous (67-68).

Several investigators have identified COMP in serum as a predictor of OA disease state and progression, suggesting that serum COMP may serve as a type 0 biomarker for OA (69-73). In addition to the examples provided here in the form of type II collagen, aggrecan and COMP, other proteins in cartilage and joint tissues may serve as sources of OA biomarkers (74).

Identifying the source of the biomarker – Molecules or molecular fragments which are present in joint fluid, and which we know are normally resident in e.g. joint cartilage may be generated primarily from the cartilage of the joint (Figure 3). However, this assumption relies on the molecule being significantly more abundant in healthy or arthritic cartilage than in any other joint tissue, or that its metabolic rate in cartilage is much higher than in other joint tissues. Comparisons within patients of joint fluid versus serum concentrations of a marker may help in determining the source. COMP, an OA biomarker of continued interest, may serve as an example: the total mass of COMP in the menisci of the knee may approach that in the joint cartilage of the knee (75), and COMP is also present in other joint tissues (76). COMP is produced in increased amounts in OA cartilage (74, 77) but it is also synthesized by synovial cells exposed to interleukin-1 and serum levels are related to synovitis in OA (24). Therefore, while being of considerable interest as an OA biomarker, its significance as a marker of a specific event or process in a specific joint tissue remains unclear. The source of the molecule or molecular fragment of interest may thus not always be evident, and is often more complex than originally proposed.

The process of production or source of a molecular fragment needs to be considered even when the biomarker is the product of a specific proteolytic event. For example, fragments could result from the degradation of a newly synthesized matrix molecule which has not yet been incorporated into a functional matrix, a molecule recently incorporated into cell-associated matrix, or be derived from a resident matrix molecule which is a critical functional part of the mature matrix (Figure 7). The consequences for cartilage function may differ. In general, markers are not specific for these processes, perhaps with the exception of some collagen II and aggrecan biomarkers. Specific neoepitope-containing degradation fragments containing collagen crosslinks are likely specific for the degradation and loss of “mature”, crosslinked, functional type II collagen from the tissue matrix (41, 49, 78-79). In contrast, other type II collagen

fragments not containing crosslinks may result from degradation of newly synthesized or mature collagen.

Poole and colleagues have developed antibodies and assays that recognize terminal sequences released when tissue collagenases have cleaved type II collagen chains (80-82). These antibodies have been particularly useful in detecting sites of collagen degradation in tissue sections from animal and human joints. Immunoassays based on such antibodies have also been applied to synovial fluid (50) and other body fluids (81). A sandwich (2-site) assay that targets the collagenase neoepitope from type II collagen has also been reported which applied to urine could distinguish OA patients from controls (79). Body fluid assays based on antibodies recognizing other epitopes in the collagen II triple-helical domain have also been described, including a recent report of immunoassays for a site of tyrosine nitration as a marker of the side-products of inflammation (83).

Assay of low-abundance epitopes associated with altered sulfation of chondroitin sulfate on aggrecan may help to identify newly synthesized aggrecan molecules, while assay of the C-terminal type II collagen propeptide may reflect type II collagen synthesis (60, 84-85).

Identifying the specific source of the biomarker molecule or fragment can be a problem with regard to both process and tissue. An increased rate of release of a marker may occur as a result of a net increase in degradation (resulting in net loss from tissue), or as a result of an increased rate of degradation in the presence of an increased rate of synthesis. We therefore need biomarkers specific for both degradative and synthetic events in the joint. An example of the former is the cleavage of type II collagen discussed above, and of the latter the synthesis of type II procollagen where the release of the C-propeptide reflects type II collagen synthesis (86-87).

While 'snap-shot' values of biomarkers are often used to compare with other outcomes, such as loss of joint cartilage by radiography, it is possible that measuring the "area under the biomarker curve" generated by several timed measurements may compare better to cumulative cartilage loss. Several lines of evidence suggest that loss of cartilage in OA may not be a linear but an episodic process. If so, then biomarker

measurements coinciding with or preceding a ‘loss phase’ may be able to predict or monitor these episodes. Some recent reports support this concept (27, 69).

Biomarker influences from variables other than OA – Serum COMP levels were shown to be influenced by ethnicity and sex (71, 88). Several other recent publications have shown that biomarkers levels are influenced by factors such as body mass index, diurnal rhythm, and physical activity (85, 88-90). Too little is yet known of the effects of these and other variables such as co-morbidities, medications or food intake, and how they may influence the utility of individual OA biomarkers.

It may be concluded that an estimate of the precise degradation rate of cartilage matrix in OA, based on assay of biomarkers, is very difficult to achieve and that the changes monitored are relative at best.

Potential uses of current generation of OA biomarkers

Roles of biomarkers outside formal classification of types 0-II – Biomarkers may be useful beyond the formal type 0, I and II definitions, for example in studying OA *disease dynamics and pathogenesis*. The temporal changes in joint fluid concentrations of fragments of aggrecan, cartilage oligomeric matrix protein (COMP), type II collagen, bone sialoprotein, MMP-3, and MMP-1 after joint injury and in developing OA are consistent with the changes in metabolic rate observed for these molecules in animal models in vivo and in human osteoarthritic cartilage in vitro and with increased human subchondral bone turnover in vivo (25-26, 60, 86-87, 91-92). Of particular significance here are the findings of type II collagen degradation early in the disease development pointing to a potential treatment target.

Structural analysis of molecules and their fragments released from or remaining in the cartilage matrix can yield useful information on matrix turnover, the protease(s) responsible and so help *identify molecular treatment targets*. Some results obtained with aggrecan fragments may serve as an example. The aggrecan core protein contains multiple potential proteolytic cleavage sites and the molecular population contained in the cartilage matrix is heterogeneous with evidence of gradual C-terminal trimming of the core protein. This confounded the search for the key proteases responsible for aggrecan release from cartilage in joint disease. However, structural analysis of

aggrecan fragments from human joint fluid showed that the major N-terminal sequence was consistent with activity of an aggrecanase (61-62). Subsequent work leading to the identification and cloning of aggrecanases (ADAMTS-4, -5), demonstrated a key role for ADAMTS-5 in a mouse model for OA and identified it as a potential treatment target in human OA and other joint disease (64-65). Assays specific for aggrecan proteolytic neoepitopes (see above) will be important in establishing the role of ADAMTS-5 in human OA, and screening during early stages of drug development (Figure 6). In this example of candidate target development and validation, biomarkers may aid in *bridging between animal models and human phase I studies*.

A marker of disease severity, reflecting underlying pathogenic mechanisms and predicting clinical outcome independent of treatment is defined as a *type 0 biomarker*. In OA, structural disease severity (stage) is traditionally measured by the Kellgren and Lawrence grade of radiological changes (which measures joint remodeling as well as destruction) or by the amount of cartilage loss measured at arthroscopy or by MRI. Interestingly, a recent study using MRI documented a relationship between knee cartilage damage measured by this technique and the molecular biomarkers serum COMP and a type II collagen cleavage neoepitope (93). This is promising in terms of understanding processes leading to OA biomarker changes, but at this stage it is simply a relationship between one biomarker and another; neither of them yet representing a true surrogate OA outcome or clinical outcome.

Several assays of molecular markers developed for patients with arthritis have been promoted as *prognostic markers* and tested to see whether they predict the onset or progression of OA. For example, levels of serum hyaluronan have in some studies on patients with clinically diagnosed knee OA predicted subsequent progression of knee OA (20-23). An increase in serum COMP has in several reports been shown to predict subsequent radiographic progression of OA (69-70, 94). Consistent with a relationship between serum COMP levels and synovial inflammation (24), increased serum levels of CRP were associated with OA progression (28, 95). Plasma levels of MMP-3 predicted radiographic knee joint space narrowing over 16 months in a prospective, longitudinal OA clinical trial (27). Finally, increased levels in urine of type II collagen C-telopeptide crosslinked peptides (CTxII) correlated with OA disease load and subsequent disease progression (54, 96). Such biomarkers show the promise of *predicting OA progression*,

selecting study populations in clinical trials, and identifying those that may best benefit from treatment.

For all currently explored OA biomarkers, a considerable overlap exists between affected and non-affected individuals, and between those with disease progress and those without. Interpretation of results is further confounded by the fact that most comparisons between groups are cross-sectional and retrospective. Results to date are best considered as hypothesis-generating, and will require confirmation in large longitudinal, prospective studies such as the OsteoArthritis Initiative.

The *evaluative test*, on the other hand, focuses on the ability of a marker to monitor change over time in the individual patient, often expressed as sensitivity to change or effect size. The effect size is dependent on the amount of change for the test, divided by the baseline variation in the test. Knowledge of longitudinal, within-patient variability and correlations with other measures of disease activity is thus important, although there are only few published studies (97). Molecular markers that can *monitor a response to therapy in OA*, may be valuable as sensitive surrogate measures of outcome in therapeutic trials, in the ideal case providing “early warning” and indications of clinical outcome. Before then we need to learn more about disease mechanisms and the release of molecular markers at the tissue level from cartilage and other joint tissues. The current lack of disease-modifying treatments in OA is a major barrier to marker validation and new randomized, controlled clinical trials will provide important opportunities.

Given that markers reflect the dynamic state of cartilage or bone metabolism, it is likely that markers will be used clinically to evaluate the dynamic changes in disease, as prognostic tools to identify those at high risk of rapid progression, or as measures of response to treatment, to identify the responders and assess the degree of response. Other potential uses of markers (e.g. as diagnostic tests) seem less likely.

Bone biomarkers and osteoporosis drug development – a role model for osteoarthritis?

Approved commercial assays for biochemical markers of bone formation and bone resorption in serum and urine have been widely used to assess disease activity and

responses to therapy in osteoporosis (OP), particularly in drug trials. They represent indices of bone turnover rate and the dynamic balance between resorption and formation of bone matrix, which is also altered in OA. Comparisons of different assays that superficially measure the same type of bone activity such as resorption, reveal consistent differences in responsiveness, and hence discriminant validity, dependent on the clinical condition. Some markers are measured with more precision than others; others may be more specific for bone of a particular quality and yet others may have variable rates of metabolism by liver and other organs and therefore not always accurately reflect bone turnover. Despite these limitations, bone marker studies have been used to identify women with and at risk for OP, to detect high bone turnover states, and to follow treatment, especially with anti-resorptive therapy (98).

In transferring the concepts of osteoporosis to OA, several issues need to be considered: (a) Alterations in subchondral bone turnover in OA, while not necessarily systemic as in osteoporosis, can be measured in serum and urine (99-101). Cartilage turnover in a single joint may not be easily detected in serum and may require synovial fluid sampling (see discussion above). (b) Variations in clearance from synovial fluid caused by varying degrees of inflammation is a concern. (c) As discussed in other sections of this chapter, practical barriers will make it harder to validate the clinical usefulness of markers in OA compared to osteoporosis. Obtaining synovial fluid is more difficult, and having access to control subjects to assess normal levels of markers in synovial fluid in non-diseased joints is difficult. Serum levels would be much easier to determine in normals, but results on serum may be harder to interpret. An example is the reduction in serum levels of the C-propeptide of type II collagen in patients with OA, despite the elevations seen in OA cartilage (87). (d) Precise and accurate bone mineral density assessments in osteoporosis that provide a well-defined endpoint have provided a consensus 'gold standard' used in patient diagnosis, management and drug development as well as biomarker evaluation. Similar quantitative measures of disease status in an OA joint are not yet possible. Markers may therefore be more difficult to validate and use clinically in OA than they have been in osteoporosis. (e) A further obvious hindrance in OA is the lack of agents that can predictably and reproducibly alter the metabolism of joint cartilage, whereas PTH and other hormones, steroids and

bisphosphonates can produce clear changes in bone turnover that can be used to explore the utility of bone biomarkers.

Barriers to validating OA biomarkers as surrogate endpoints

Validating biomarkers as surrogate endpoints is a demanding process, as discussed in previous reviews (14-15). Aspects of a prognostic OA marker (type 0) are discussed here as an example. First, a prognostic marker should have a biological rationale. The marker should be identifiable in and highly specific for the target tissue such as cartilage or bone or be a protease, cytokine or growth factor or some other such molecule. The role of the marker in the pathogenic pathway should be understood. Second, a prognostic marker measured at baseline in a body fluid should correlate with later patient-relevant changes. If the level of a marker is abnormal at baseline, then the risk of subsequent joint deterioration may be magnified. The course of OA should be evaluated by accepted measures that are independent from the marker. Third, the marker should be detectable in all OA patients and should correlate with appropriate measures of disease dynamics if these are available. Fourth, the validation process needs to include samples from patients with a spectrum of OA severity and range of potentially interacting variables. Fifth, any biomarker, surrogate or not, needs to be measured by reliable methods described in sufficient detail to be replicable by others. This latter issue is discussed in detail in recent guidelines on standards of diagnostic accuracy and reporting (102-103). It is possible that a combination of markers, or marker ratios, will prove to be more useful than a single marker (104-108).

Validating a biomarker as a surrogate endpoint/measure is complex. In addition to testing how well a putative surrogate endpoint reflects patient preference and quality of life, how well the marker responds to adverse effects that may overshadow the apparent benefit, is equally important. Further, even if a surrogate endpoint/measure is identified and validated, beneficial effects may occur via pathways that do not include the surrogate.

For all marker applications, but in particular in monitoring treatment response, it is essential to establish the variability over time and between individuals in representative and stable cohorts of appropriate size (97). Such data can be used in power analyses to

calculate the required number of patients and the required response to treatment in a clinical trial setting.

The promise that broad screens can identify new OA biomarkers

Most of the OA-related work on biomarkers has thus far taken the ‘candidate protein’ approach of exploring changes in body fluids of proteins or protein fragments with a known or suspected function in joint cartilage. While several promising candidate markers have thus been identified, this approach has its limitations. It may be argued that the search for OA biomarkers needs to expand to be genome-, transcriptome-, proteome-, and metabolome-wide and accelerated by large-scale screening techniques as used in proteomic and gene-expression profiling, using joint tissues and circulating blood cells. For continued biomarker research using either traditional or newer approaches, access to large biological specimen repositories linked to high quality longitudinal clinical data is critical. This may, in the face of the slow natural history progress of OA, be the most difficult limiting factor.

As an example of a broad screening approach, high resolution nuclear magnetic resonance (NMR) spectroscopy tuned to examine body fluids for differences in low molecular weight metabolites revealed evidence that markers of lipid metabolism are consistently altered in animals with OA (109-110). Another example of a related methodology is the use of ¹H-NMR serum spectroscopy and multivariate statistics to detect epithelial ovarian cancer with 100 % sensitivity and specificity (111). Such technology combined with access to large, well characterized sample collections as generated in the OsteoArthritis Initiative may improve the odds of success in the search for OA biomarkers.

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Figure legends

- Figure 1 Pathogenic mechanisms of OA. Endogenous factors such as age, genetics and obesity interact with environmental factors influencing joint biomechanics and load. Factors that drive changes in joint structure are not necessarily the same factors that drive symptoms and pain. Joint pain is possibly enhanced by inflammation, and pain may drive inflammation. Modified from (2).
- Figure 2 What subjects should be selected for OA studies? A study could examine a random sample of the entire population, or subjects with or without OA. Should the subjects selected for study of potential disease modification or prevention be those who are asymptomatic and do not yet have radiographic changes but who, for some reason, are at high risk of developing OA in the future (corresponding to the bottom center circle)? Or should we select subgroups of subjects who have symptoms of OA but no radiographic changes, or those who have radiographic changes but no symptoms? We do not know how many persons fit into each of these categories or what is the “conversion rate” to “OA classic” (“Both”). What proportion will convert in a given time period? The choice of study population determines the conclusions possible to draw from a biomarker validation study. Modified from (2).
- Figure 3 Biomarker concentrations in body fluid compartments are influenced by many processes. Modified from (16).
- Figure 4 The cross-linking amino acids, pyridinoline (Pyd) and especially deoxypyridinoline (Dpd) in urine were found to be more effective markers of bone resorption than hydroxyproline. In the last decade, the small telopeptide fragments NTx and CTx, measured by immunoassay, have proved to be even more sensitive and convenient markers of bone resorption. Their release from bone and excretion by the kidney is believed to depend on the osteoclastic degradation of bone collagen directly to low molecular weight peptides as shown in the cathepsin-K mediated path on the left.

- Figure 5 Collagen peptide epitopes as biomarkers of bone and cartilage degradation. In measuring bone resorption, the cross-linked telopeptide markers NTx and CTx, from osteoclastic breakdown of type I collagen, can be assayed in urine. Similarly, CTx II a cross-linked C-telopeptide from type II collagen, can be assayed in urine. It probably represents an index of mineralized type II degraded by osteoclasts (Lohmander et al. 2003). The type II collagen telopeptide, NTx II, is theoretically another marker of type II collagen breakdown in the same category. In addition antibodies and assays against the neoepitopes created by collagenase are in use.
- Figure 6 Aggrecan degradation and generation of neoepitopes. Several assays exist for the detection of aggrecan fragments in body fluids. However, so far they lack specificity for the different possible proteolytic pathways degrading aggrecan. For example, assays that rely on dye precipitation will detect all fragments containing chondroitin sulfate or keratan sulfate (CS, KS), irrespective of the proteolytic cleavage site. The availability of assays specific for the proteolytic neoepitopes indicated (ARGS, ITEGE, FFGV, DIPEN, SELE, KEEE), or others, would make it possible to monitor specifically the activity of e.g. the aggrecanase or matrix metalloproteinase pathways.
- Figure 7 Fragments of matrix molecules may be generated by degradation of: newly synthesized molecules which are never incorporated into the functional matrix, molecules which have recently been incorporated into a functional matrix, and molecules which have been long-time members of the 'resident' functional matrix. It is not always possible to distinguish these different sources of fragments. Another source of heterogeneity is the origin of fragments from pericellular, territorial, and interterritorial matrix, as well as from superficial and deep layers of the joint cartilage. Modified from (92).

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