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Biochemical markers and the FDA Critical Path: How biomarkers may contribute to the understanding of pathophysiology and provide unique and necessary tools for drug development

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Abstract

The aim of this review is to discuss the potential usefulness of a novel class of biochemical markers, neoepitopes, in the context of the US Food and Drug Administration (FDA) Critical Path Initiative, which emphasizes biomarkers of safety and efficacy as areas of pivotal interest. Examples of protein degradation fragments – neoepitopes – that have proven useful for research on bone and cartilage are collagen type I and collagen type II degradation products, respectively. These markers have utility in the translational approach, as they can be used to estimate safety and efficacy in both preclinical models and clinical settings. Biochemical markers of tissue degradation may provide optimal tools, which in combination with other techniques, prove essential to drug discovery and development.

Keywords: *Neoepitope; biomarker; FDA; critical path; translational science*

Introduction

The current costs of bringing a new drug to the market are estimated to be as high as $800 million to $1.7 billion (FDA 2004). Often, product development programs are abandoned after extensive investment of time and resources, and the high failure rate drives up costs. The path to market even for successful candidate compounds is long, costly, and inefficient, due in large part to the current reliance on cumbersome methods of assessing efficacy and safety. During the last decade, the number of new drug and biologic applications submitted to the US Food and Drug Administration (FDA) has declined significantly. As the costs and challenges of medicinal product development keep rising, innovation may continue to stagnate or decline, precluding advances in addressing a wide range of unmet health needs. In the current environment, it is essential to improve efficiency in drug development. Efficiency could be greatly improved if one could reliably predict at an early stage, which projects have the highest probability of success. There is a need for novel early-predictor methodologies – in essence, a drug development tool kit – containing powerful scientific and technical resources such as *in vitro* tests, predictive animal models, biomarkers for safety and efficacy, and new clinical evaluation techniques.

The ability to use disease models to predict human response is important in the preclinical development of drugs. Methods are needed to bridge results from disease models to the clinical setting to help ensure maximum product efficacy and safety. To achieve this

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critical goal, a combination of better animal models and tissue-specific biomarkers may be a prerequisite in some diseases. Insights gained from preclinical investigation of specific disease states may also prove bridgeable to other disorders. At later stages of development in clinical trials, there is a demand for innovative and efficient study designs with improved clinical end points, Incorporating novel, validated biomarkers of efficacy and safety in both preclinical and clinical research could improve the cost-effectiveness of the entire product development process.

Recently, three important statements relating to drug research have been released independently. They provide guidelines for the creation of a new and better drug development tool kit:

1. The FDA has articulated a Critical Path Initiative (FDA 2004) to improve the efficiency of product development industry-wide. One of its objectives is to identify and prioritize the most pressing development problems for new drugs and other therapeutic agents. In particular, the FDA Critical Path Initiative attempts to bring attention to new scientific research tools that may revolutionize the regulatory and scientific process for new product approvals. The FDA stipulates that 'there is a demand to create new tools to get fundamentally better answers about how the safety and effectiveness of new products can be demonstrated, in faster time frames, with more certainty, and at lower costs' (FDA 2004) – a new product development tool kit.

2. The recently proposed BIPED (Burden of Disease, Investigative, Prognostic, Efficacy of Intervention, Diagnostic) classification, developed by the US National Institutes of Health (NIH)-funded Osteoarthritis Biomarkers Network, has further highlighted the need for understanding and using biomarkers (Bauer et al. 2006). Importantly, emerging classes of biochemical markers that are more pathology-specific have been identified. The use of novel techniques employing specific protein degradation fragments have proven valuable for slow-progression diseases, such as osteoporosis and osteoarthritis. Lessons may be learned from these approaches that can be implemented in other diseases. Using two successes from bone and cartilage studies as examples, we describe in this review how biochemical markers may be unique assessment tools in translational science, from basic research to clinical trials, and therefore can be used as efficacy and safety makers.

3. Increased focus is directed at the apparent disconnection between the complicated pathology of an organ and the simplistic high-throughput screening (HTS) approach chosen by most drug developers. Although the reductionistic HTS approach may be needed and is driven by the desire for a simple assay capable of screening hundreds of thousands compounds, it may not be appropriate for the complicated pathology of disease. HTS may therefore lead to more false-positive candidate compounds than other approaches that take into greater account the overall tissue pathophysiology.

Recent research within the osteoarthritis field strongly suggests that the encouraging *in vitro* potencies and preclinical efficacy of new products have not translated into clinical efficacy (Spector et al. 2005, Bingham et al. 2006, Garnero et al. 2008, Murphy and Nagase, 2008, Krzeski et al. 2007). Thus, techniques and approaches which aid assessment and monitoring of the whole tissue pathology may be preferred for both screening potential targets and selecting novel ones.

Recent advances in the field of biomarkers – in particular, the use of protease-generated degradation fragments of pathologically relevant proteins, so-called neoepitopes – have been suggested to be of particular relevance in assessments of the entire tissue phenotype, and in animal and clinical studies (Schaller et al. 2005a, Leeming et al. 2006a, b, Alexandersen et al. 2007). In this review, we describe the application of new tools – biochemical markers – in innovative drug research programmes to achieve faster development of safer and more effective treatments for the benefit of patients. In addition, these markers can help identify the individuals with or without disease, those with widespread disease, and those who will benefit most from medical intervention.

We ask the specific question: 'Can we implement what is learned from osteoporosis and osteoarthritis in the search for an optimal drug-development tool kit, which may assist in future safety and efficacy evaluations across the spectrum from animal to clinical studies?' Osteoporosis is particularly well suited to this inquiry, as the field has advanced in the last decade from near-complete absence of assessment techniques and drugs to the availability of validated surrogate markers of efficacy and prognosis and a range of potential treatment options.

We specifically evaluate whether lessons learned from matrix turnover biology in osteoporosis and osteoarthritis that have resulted in both new assessment techniques and treatment opportunities may be applied to other diseases involving excessive matrix turnover. For example, the approaches derived from studies of these two slowly degenerative diseases may be extended to cardiovascular disease and fibrosis, both of which involve pathological matrix remodelling.

Finally, we speculate that this new product development tool kit and the methods for its use may be closer to realization than they appear, if the lessons from successes in assessing protease-generated fragments are garnered and applied to other diseases.

The FDA Critical Path

The costs of bringing a new drug to market have increased by a factor of 4 and the number of FDA approvals has decreased by factor of 4 over the last 20 years (FDA 2004). As the costs and difficulties of medical product development continue to grow, innovation will continue to suffer and may therefore not provide the impetus for new drugs for a wide range of currently untreatable diseases. Just as importantly, second-generation treatment opportunities for diseases with first-generation intervention strategies may not be sufficiently cost-effective to investigate and develop.

The FDA has formulated a Critical Path Initiative to improve the efficiency of product development industry-wide and to identify and prioritize: (1) the most pressing development problems for new drugs and other therapeutic agents, and (2) the areas that provide the greatest opportunities for rapid improvement and public health benefits.

Many of the novel basic science discoveries in recent years may not quickly yield more effective, more affordable, and safer medical products for patients. The obstacles to rapid progress include not only the increasing challenges, inefficiencies and costs of medical product development, but also the paucity of applied research to develop from novel ideas, the quantifiable assays and techniques which can be used to assess safety and efficacy in drug development. Basic researchers are successful in the identification of mechanisms of pathology; however, there is a growing concern that new science is not guiding the technology *development* process in the same way that it is accelerating the technology *discovery* process. As considerable effort is directed toward identifying new proteins or new functions of old proteins, less of the total research resources are available to create new tools to understand the ways in which the safety and effectiveness of new products can be demonstrated faster, with greater certainty, and at lower cost than is possible today.

This shortfall in applied science forces drug developers to use tools and concepts based on often imprecise, inaccurate or cumbersome older technologies and pathobiological understanding. This situation results in the failure of the vast majority of investigational products that enter clinical trials, sometimes at a late stage after significant investment of time and resources. Only a concerted effort to apply new biomedical science to product development will succeed in modernizing the critical path. The observation of a technological disconnection between discoveries in basic science and the product development process prompted the FDA to conclude that 'a new product development tool kit, containing powerful new scientific and technical methods, such as animal or computer-based predictive models, biomarkers for safety and effectiveness, and new clinical evaluation techniques, is urgently needed to improve predictability and efficiency along the critical path from laboratory concept to commercial product'.

Scientific efforts may in part have to be redirected to ensure that basic discoveries are transformed into new and better tools to optimize the process of developing medical treatments. An important aspect of this endeavour involves taking basic science landmarks and generating quantifiable methods of assessment that are predictive for drug efficacy and safety.

Ensuring product safety is a pivotal consideration in the design of a drug molecule. The traditional tools used to assess product safety – animal toxicology and outcomes from human studies – have changed little over many decades and have largely not benefited from recent gains in scientific knowledge. The inability to better assess and predict product safety has led to failures during clinical development and, occasionally, after marketing. Importantly, the efficacy marker in one study may indeed be the safety marker in another study.

Unproven efficacy accounts in large part for the failures that occur late in product development. Better tools are needed to identify efficacious products earlier and more efficiently in the development process. Such advancements will help protect subjects, better utilize research-and-development investment by focusing efforts on those products expected from an early stage to succeed, and bring needed treatments to patients sooner. Advanced tools may yield further benefits in concert with improvements in the efficiency and effectiveness of the clinical trial process, including the implementation of trial designs, end points and analyses with disease-specific components.

In the present context our focus is on the development and use of unique biochemical markers of tissue turnover and, to a lesser extent, advanced imaging techniques, as well as the combination of these powerful tools, to predict drug efficacy and safety.

Bridging translational science and the problems of the reductionistic approach

Predicting and subsequently demonstrating medical efficacy are among the most difficult and important challenges in pharmaceutical product development. Currently available animal models have demonstrated limited predictive value in some diseases, such as osteoarthritis (Krzeski et al. 2007). Non-clinical screening methods with greater predictive utility are urgently needed to aid in the selection of the best possible development candidates and strategies. A number of authors have raised the concern that the current drug discovery process, based as it is on *in vitro* screening techniques

and animal models of (often) poorly understood clinical relevance, is fundamentally unable to identify candidates with a high probability of effectiveness (Horrobin 2003, Duyk 2003) The current scientific understanding of both physiological and pathophysiological processes is of necessity reductionistic, existing for example, at the gene, gene expression or pathway level. It often does not extend to knowledge at the level of the systems biology of the cell, organ or whole organism, and it certainly does not reach a systems understanding of the pathophysiology of particular diseases. Possibly, approaches entailing a larger pathophysiological perspective, measuring effects on whole tissue function rather than on single enzymes and targets, may better predict final clinical outcome. Reaching this more systemic and dynamic understanding of human disease and applying screening methods that take it into account will require major additional scientific efforts focused on screening systems more complex than traditional HTS.

Nevertheless, progress in drug discovery will continue, and as candidates emerge, the best tools available should be used for their evaluation. This will require strengthening and rebuilding the relevant disciplines (e.g. physiology, pharmacology, clinical pharmacology), as well as identifying ways to bridge from the laboratory to the whole organism and correlate early markers of safety and benefit with actual outcomes in patients.

The Critical Path and biochemical markers

The FDA states, 'Additional biomarkers (quantitative measures of biological effects that provide informative links between mechanism of action and clinical effectiveness) and additional surrogate markers (quantitative measures that can predict effectiveness) are needed to guide product development'. This appears to be an open invitation for new and improved biomarkers, in particular biochemical markers.

Biomarkers may assist in most aspects of drug development, as illustrated in Figure 1. Most optimally, use of biochemical markers may extend from *in vitro* testing through animal models to clinical settings, thereby enabling researchers to better interpret efficacy and safety while maintaining a pathophysiological approach centred on whole tissue. It is likely that biomarker approaches will result in earlier opportunities for 'proof-of-concept' trials, which should be used to confirm activity in humans before a commitment to full-scale development is made. We discuss in detail these combined challenges and opportunities in the following sections.

Bone and cartilage – the challenge of slow progression

Osteoporosis (OP) and degenerative joint diseases (DJD) – such as osteoarthritis (OA) and rheumatoid arthritis (RA) – remain major and growing epidemiological problems worldwide. Because of rising longevity in the elderly populations of industrialized countries, the number of persons requiring medical attention and ultimately treatment is continuously increasing (Rodan & Martin 2000). OP has an estimated prevalence of 40% in Caucasian women over 80 years old, whereas OA and RA affect 12% and 1%, respectively, of the US population (Lozada & Altman 1999, Olsen & Stein 2004).

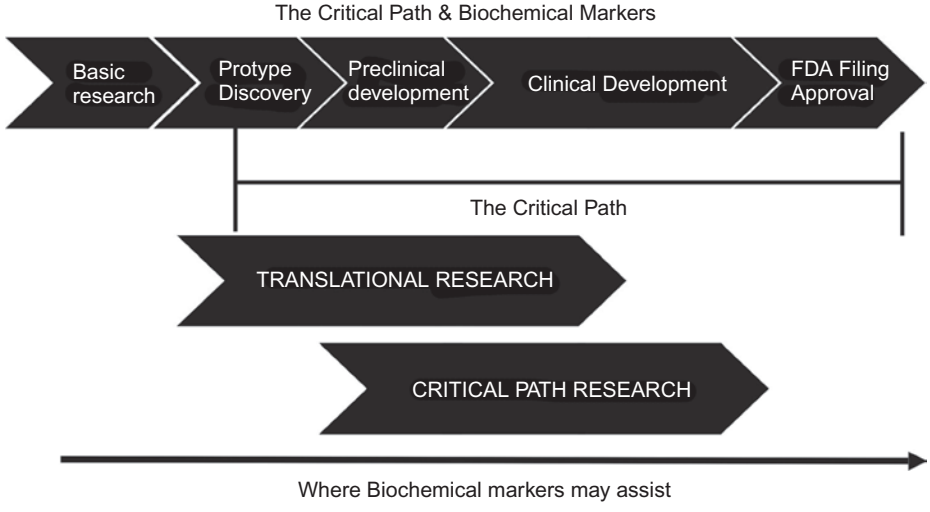

Figure 1. The FDA Critical Path: the bottom arrow indicates that biochemical markers may be useful at all steps of research in the critical path. Modified from FDA (2004).

OP and OA are characterized by slow progression. Slowly progressive diseases pose a range of drug development challenges, particularly in phase II dose-finding studies, and these challenges remain to be adequately solved. Because disease pathogenesis is slow, measuring progression is time-consuming and costly. Basic and clinical research of the past decade has been instrumental in clarifying some of the major mechanisms underlying the pathogenesis of OP and OA; however, much remains to be achieved before curative treatments can be provided to patients.

Biochemical markers of bone and cartilage: Need for faster and more sensitive responses

Without sensitive and reliable diagnostic methods allowing for quantification of pharmacodynamic effects, drug development faces major limitations. Numerous innovations have yielded a range of useful techniques and experimental models permitting investigation at the cellular, organ and whole-organism levels. These techniques have been instrumental in illuminating the now widely accepted concept that the common denominator in the pathophysiology of both OP and DJD is a negative balance between tissue formation and degradation (Schaller et al. 2005a). A paramount discovery was that this balance could be measured by neoepitopes – the degradation products of the extracellular matrix – and the formation fragments of the incorporated matrix proteins, and that these biomarkers could assist in the experimental or clinical monitoring of the pathogenic processes. The dynamic nature of these biomarkers, which are continuously produced and secreted, may make them optimal for monitoring disease progression and response to therapy.

During the past decade, a wide array of biochemical assays have been developed to estimate bone turnover and bone quality in the *in vitro, ex vivo* and *in vivo* settings. These assays not only facilitated the clarification of important mechanisms of skeletal homeostasis but also proved to be useful tools for evaluating the efficacy of bone-sparing drugs. Several authoritative reviews of the existing biomarkers for bone and cartilage have been published recently (Schaller et al. 2005a, Lesko & Atkinson 2001, Lathia 2002, Wagner 2002, Frank & Hargreaves 2003, Leeming et al. 2006a, b, Sumer et al. 2006, Bauer et al. 2006), individual discussion of which lies beyond the scope of this paper.

During the past two decades, the situation in OP has progressed from an absence of diagnostic, prognostic and efficacy-assessment techniques to the availability of a basket of validated techniques and treatment options. However, the OA field has yet to meet many of these challenges. The substantial lag in clinical achievements in the field of OA highlights the need for a similar battery of techniques to assist in the evaluation of potential chondroprotective agents. There is a need for surrogate markers offering accurate, precise and fast responses in the development of drugs for diseases of slow progression.

Diagnosis and monitoring of osteoporosis and osteoarthritis: need for fast and sensitive responses

The diagnosis of OP is inherently linked to low bone mass, and this surrogate end point is well integrated into the development of new anti-osteoporotic drugs. Bone mineral content and bone mineral density are non-invasively quantified by dual-energy X-ray absorptiometry. Substantial evidence supports the paradigm that low bone mass is accompanied by enhanced bone fragility, which poses a risk for fractures (Johnell et al. 2005, Cummings et al. 2002, Melton et al. 1993). The simultaneous use of a biochemical marker can aid in the identification of osteopenic patients with negative bone balance (bone loss) who are also at a high risk for fracture and therefore should be treated. Biochemical markers bring added value to the evaluation of bone quality (Karsdal et al. 2006, Byrjalsen et al. 2007).

The diagnosis of OA is generally based on clinical and radiographic changes, which occur fairly late in the disease course and have poor sensitivity for monitoring disease progression. Progression of joint damage is likely to result primarily from an imbalance between cartilage degradation and repair, so that measuring markers of these processes would seem a promising approach to improving predictability of disease progression at the individual level. For OA diagnosis, the currently applied 'gold standard' is change in joint space width (JSW) measured on consecutive radiographs (Reijman et al. 2004). While radiographs do not allow direct visualization of articular cartilage, they provide an indirect measure of cartilage thickness. Yet radiographically demonstrated effects are considered a valid end point in trials of anti-arthritic drugs and are accepted by the regulatory authorities.

Importantly, a disadvantage of a surrogate radiography-based end point is that spontaneous annual changes in the joint as well as changes induced by medical interventions are rather small and cannot be detected reliably by current measurements. The lack of fully effective, chondroprotective medications has limited the use of potential imaging markers to monitor the effect of treatment for OA. However, owing to their dynamic changes in response to treatment, biological markers might provide relevant information more

rapidly than imaging techniques and should contribute to our understanding of mechanisms that underlie the clinical efficacy of OA treatments.

In contrast to imaging techniques, biochemical markers of bone and cartilage turnover, obtained in serum or urine samples, show changes in a markedly larger range compared with the imprecision of the assay (<8–10%). Typically for the biomarkers, a decrease of 50–80% or an increase of 100–200% in changes in the biochemical markers is observed shortly (days to weeks) after initiation of treatment with anti-resorptive or anabolic drugs (Schaller et al. 2005a). The respective changes in bone mass range from 6–7% after 2 years of bisphosphonate therapy (Hosking et al. 1998) to 2–3% or less for selective estrogen receptor modulator (SERM) agents and calcitonin (Delmas 1997, Overgaard et al. 1989), a fairly small increment relative to the precision error of 1–2% for bone mineral density (BMD) measurements. Similarly, annual changes in JSW in untreated women are in the range of 0.1–0. 2 mm (Reijman et al. 2004), which is also close to the range of the precision error of repeated measurements. Thus, biochemical markers have a more suitable signal-to-noise ratio, which may prove particularly useful in phase II studies designed to define the optimal dose of a drug. In this context, studies indicate that 3- to 6-month changes in biomarker levels are highly correlated with 2-year changes in BMD, suggesting that phase II development could be shortened considerably by the use of biomarkers (Greenspan et al. 1998, Ravn et al. 1999 a, b, Grados et al. 2003, Schaller et al. 2005a). Combinations of different types of markers – for instance, dynamic markers of tissue turnover (typically, biochemical markers) and static markers of current tissue condition (BMD by X-ray or cartilage thickness by magnetic resonance imaging (MRI)) – have provided complementary information (Figure 2) and, consequently, superior accuracy in identifying persons with progressive disease for the purposes of clinical trials (Ravn et al. 1999a, b, 2000, 2003). A simple analogy suggests the usefulness of combining information from static and dynamic markers: predicting when a bathtub will run dry depends on both the current state of the water in the tub (volume) and the current change (flow in/out). The two different types of biomarkers and how they most optimally may be combined to predict both volume and flow are discussed in subsequent sections.

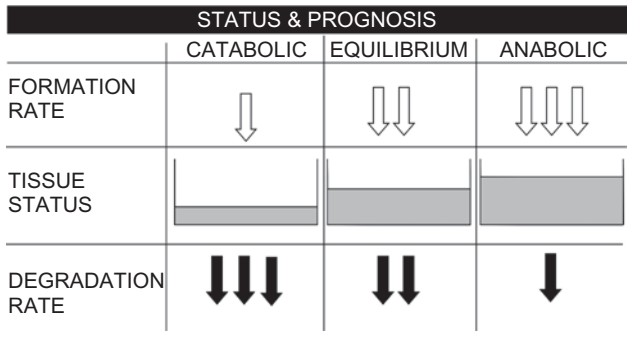

Figure 2. Schematic illustration of formation and degradation rates leading to either more tissue or less tissue depending on the balance between tissue formation and tissue degradation, i.e. whether the tissue is in a catabolic or anabolic state. Tissue condition measured by BMD, for example, provides a snapshot of tissue status, whereas assessment of biochemical markers of tissue formation and degradation provide information on change. Therefore, combining both types of assessment may provide better prognostic value.

Need for biomarkers in drug development

The overall goal of using biomarkers in drug development is to improve decision-making with respect to dosing, treatment time, trial design, risk/benefit ratio, transfer knowledge to label and ultimately save costs. Less than 10% of all drug candidates that enter preclinical studies complete clinical development and are submitted for regulatory approval. The main reasons for failure are lack of efficacy and unacceptable toxicity. By implementing biomarkers for screening of drug candidates at early time points (e.g. *in vitro* and/or preclinically), investigators can address potential safety issues in a timely way and hence increase efficiency and reduce overall costs. Drug candidates associated with safety concerns as a result of preclinical biomarker screening could be excluded from clinical trials, prompting more rapid allocation of resources to other projects with higher chances of success. Similarly, biochemical markers of efficacy, used in both preclinical studies and early clinical trials (pre-phase II), may aid in the selection of the optimal drugs or approach for full-scale development programmes.

Different types of biomarkers are needed for different stages of drug development: (1) diagnostic biomarkers enabling identification of persons with the disease in question, (2) prognostic and burden-of-disease markers, enabling identification of patients with fast progression and widespread disease, (3) allowing for selection of those most likely to benefit from treatment, and (4) biomarkers that demonstrate the progression of disease and correlate with known clinical indices (e.g. current 'gold standards').

The primary objective of biomarker use in drug development is to provide the best evidence for rejecting the null hypothesis of no treatment effect and thereby help demonstrate the efficacy of a drug candidate. Because biomarkers are considered objective measures of pathogenic or pharmacological events, the probability of type I (false-positive) and type II (false-negative) errors becomes lower. Consequently, the power calculations result in smaller sample sizes. This fits well with the 3R concept of Reduction, Refinement and Replacement for

animals in preclinical studies. However, animal models often represent a homogeneous population because of breeding procedures, whereas patient populations are far more heterogeneous. At present, biomarkers can to some degree identify individuals of interest in the general population, but greater focus must be placed on the study of population heterogeneity, because the number of type I and type II errors remains high in clinical trials.

Preclinical experimental findings might not translate into effects in humans, and therefore biomarkers should be continuously developed and cross-validated so that in combination with other preclinical efficacy assessments they can depict actual patient responses. Validated biomarkers of specific physiological mechanisms and efficacy can facilitate rapid progress from phase I to phase II clinical studies on the basis of the exposure–effect relationship, giving researchers confidence in the maintenance of efficacy in the translation from mice to man. Beyond the drug development process, these biomarkers may optimally be used in the assessment of the individual patient's response to treatment. By evaluating the results of biomarker assays, clinicians may be able to determine, for example, whether a therapy has had the desired effect, the drug dose or treatment regimen should be changed, disease progression has occurred as predicted, or a new end point has occurred. Currently, only a few validated and approved biomarkers are available for use in preclinical studies and clinical trials. There is an urgent demand for more focus on and research in the field of biomarkers.

BIPED – classification and needed validation of biomarkers

If biomarkers are to be used extensively in drug development, consensus validation and understanding of their potential advantages and drawbacks are necessary. Obviously, the higher the threshold for validation, the more confident workers in other areas may feel about the use of these markers. This presents a dilemma, however, as there is an immediate need for the use of markers in several fields.

One approach to the validation of biochemical makers was recently proposed by Bauer and colleagues (2006), representing the NIH-funded OA Biomarkers Network, a multidisciplinary group interested in the development and validation of OA biomarkers. This group proposed the BIPED biomarker classification (Table 1 and Figure 3). It also offered suggestions on optimal study design and analytical methods for use in OA investigations. The BIPED classification provides specific biomarker definitions, with the goal of improving capabilities for the development and analysis of OA biomarkers and of communicating advances within a common framework.

The development and assessment of longitudinal (prognostic and efficacy) and cross-sectional (diagnostic and burden of disease) markers require validation with well-designed case–control studies. Many of these markers are dependent on normal biological variation; it is therefore important that personal data are collected, such as sex, age, disease severity and treatment history. The purpose of the diagnostic marker would be to distinguish between different states – e.g. presence and absence of disease – within the general population. Burden-of-disease markers should be able to distinguish severity or extent of disease within the group that is positive for the diagnostic marker. A prognostic marker ideally could predict outcome from a given baseline situation of a patient through calculation of the relative risk (RR) and odds ratio (OR). It is consequently important that in the development process, the biomarker is compared with one or more 'gold standards'. In OA, the gold standard is joint space narrowing (JSN) or JSW, which in many incidences is combined with a more qualitative assessment, such as radiography (e.g. Kellgren–Lawrence (KL) scoring). Both diagnostic and prognostic biomarkers should optimally have high sensitivity and specificity for differentiation of subjects with and without disease. However, this is where many of the existing biomarkers tend to fail. In numerous studies, two cohorts have been found to differ significantly in mean levels of a given biomarker, but the biomarker failed to identify a relatively high percentage of the individual patients as belonging to one cohort or the other. In reality, a panel of markers is needed for predicting disease course for the individual patient.

Table 1. Description for the BIPED classification. Extracted from Bauer et al. (2006).

Burden of disease:	Burden-of-disease markers assess the severity or extent of disease, typically at a single point in time, among individuals with OA.
Investigative:	An investigative marker lacks sufficient information to allow for its inclusion in one of the existing biomarker categories. The investigative category includes markers for which a relationship to various normal and abnormal parameters of cartilage extracellular matrix turnover has not yet been established in human subjects.
Prognostic:	The key feature of a prognostic marker is the ability to predict the future onset of OA among persons without OA at baseline or the progression of OA among those with the disease.
Efficacy of intervention:	An efficacy-of-intervention biomarker provides information about the efficacy of treatment among persons with OA or those at high risk for its development.
Diagnostic:	Diagnostic markers are defined by the ability to classify individuals as either having or not having a disease.

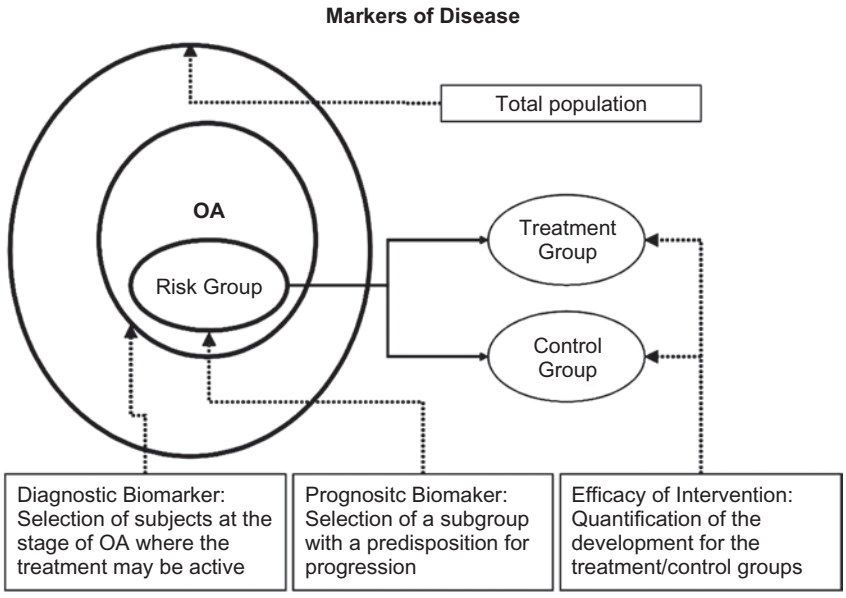

Figure 3. The three major categories of the BIPED classification, illustrating the types of uses of biomarkers. Modified from Bauer et al. (2006).

Advantages of biochemical markers in clinical settings

In a clinical trial setting, diagnostic and prognostic markers may optimally be used to identify patients with the disease of interest and thereafter those having a high risk of progression. Later, the effect of a potential treatment may be monitored by a marker of efficacy, as described in Figure 3. Biochemical markers have several advantages in clinical trials: (1) the collection of samples is non-invasive and therefore can be repeated continuously over a long study period, facilitating longitudinal prospective studies; (2) test results can be obtained quickly, enabling disease progression to be monitored continuously and identification of responders and non-responders to occur earlier than is possible with less dynamic measurements; (3) patients able to see treatment efficacy by means of surrogate markers may be encouraged to continue in the trial, thus potentially avoiding some of the problems surrounding compliance with therapy in chronic and even life-threatening diseases; (4) a relatively small sample size may be required for completing a clinical trial because of a more favourable sensitivity to change in relation to the coefficient of variation, compared with other methods, which is essential in power calculations; (5) validated diagnostic and prognostic biomarkers may aid in new drug development in both the preclinical and clinical phases, by identification of positive effects of already known treatment for other indications; (6) biomarkers identified for one disease might be applicable to another as safety markers, as described in the Critical Path. Such applications are further discussed in subsequent sections.

Neoepitopes – novel opportunities

One class of biomarkers comprises those measured in blood or urine. Important research in this area has aimed at the identification of proteins of relevance to pathology, which might be surrogates of whole-tissue function. One approach to identifying pathologically relevant proteins is to combine tissue-specific proteins and the pathological expression of some proteolytic enzymes. The action of enzymes on extracellular matrix (ECM) components results in matrix degradation fragments, or neoepitopes. This class of biomarkers has proven successful in studies of OP and OA, and lessons learned in these diseases may be applied to other disease conditions.

The most abundant molecules in the cartilage ECM are collagen type II and aggrecan. These proteins are sequentially degraded when cartilage erosion occurs in either RA or OA. In OA, the tissue of interest is the articular cartilage (Figure 4). The enzymes presently receiving the most attention are the matrix metalloproteinases (MMP) and aggrecanases (ADAM-TS). Protease-generated fragments of collagen type II and aggrecan produced by these important enzymes may be relevant molecules in cartilage destruction.

Pathologically relevant protein modification is not restricted to protease activity, although the subpopulation of neoepitopes generated through this mechanism may be of paramount importance in relation to the critical path and the use of biochemical markers in drug discovery and development. Important post-translational modifications that may also be pathology relevant

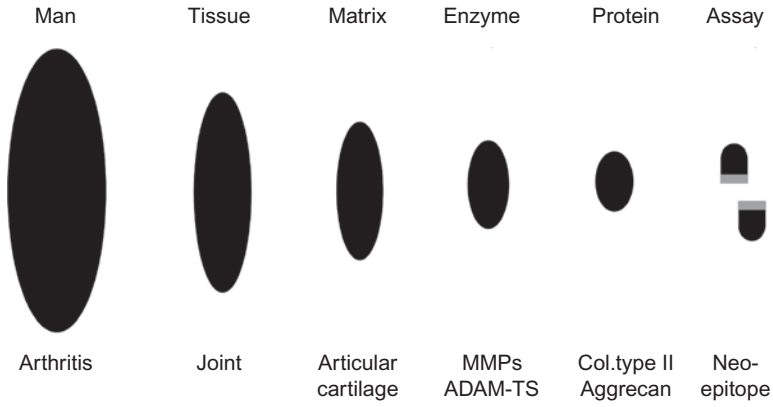

Figure 4. Visualization of the generation of pathology-relevant neoepitopes. In OA, the areas of interest are the joints. The tissue of interest is the articular cartilage. The enzymes presently receiving the most attention are the matrix metalloproteinases (MMP) and aggrecanases (ADAM-TS). The most abundant cartilage proteins are collagen type II and aggrecan. Protease-generated fragments of collagen type II and aggrecan produced through the action of these important enzymes, which may be relevant molecules in tissue destruction, can be used to monitor tissue turnover. These fragments, such as C-terminal telopeptide of type II collagen (CTX-II), may be used in clinical settings, in preclinical models and in simple *ex vivo* and *in vitro* systems. Therefore, neoepitopes may be excellent candidates for translational science.

include hydroxylation of prolines, resulting in hydroxyprolines, cleavage of procollagen peptides, cross-linking both intramolecular and intermolecular collagen fibrils (Bailey & Peach 1968, Oxlund et al. 1995, Bank et al. 1999, Mercer et al. 2003, Avery & Bailey 2005), age-related changes, such as isomerization and glycation (Fledelius et al. 1997, Brady et al. 1999, Avery & Bailey 2005), resulting in iso-aspartic acids and advanced glycation end products such as pentosidine (Bailey et al. 1998, Sell & Monnier 1989, Avery & Bailey 2005) and nitrosylation of tyrosines.

A range of protease-generated neoepitopes has already been described in the literature, but they have not been utilized by applied science to produce quantifiable methods of disease assessment. In the context of bone and cartilage, collagen type I and II as well as aggrecan are the most interesting and best described. Figure 5 provides a schematic representation of the important protease degradation sites in these proteins. Assays detecting a few neoepitopes have been developed and are used in both clinical and preclinical studies.

The neoepitope approach appears promising for the development of pathology-specific biochemical markers. However, a range of considerations are relevant when assessment technologies, such as enzyme immune assays (EIA), are developed. Biochemical markers conforming with the FDA Critical Path should be usable in *in vitro* studies, preclinical science and human clinical trials. This requires a range of features (e.g. applicability across species), some of which are summarized in Table 2.

Table 2. Challenges encountered during development of biochemical markers.

Topic	Challenge	Solution	Comment
Species	Assay to be used in translational, (a suggestion) cross-species research	Analyte target sequence to be preserved in as many species as possible	Antibodies may be difficult to elicit for the EIA because of low immunogenicity
Protein	Target tissue specificity	Attain specificity by target-tissue analyte characteristics (e.g. neoepitopes)	Two distinct neoepitopes can be combined in one sandwich ELISA such as CTX-I (Schaller et al. 2005a)
Target-tissue profile	Assay to be used for whole organ tissue pathophysiology	Selection of epitope generated by a range of similarly regulated proteases within target tissue	Protease pan-family-specific biomarkers may respond less well to intervention
Sample material	Analyte measurable in easily obtained body fluid	Selection of analyte that is present in serum/plasma or urine	Compartment distribution and kinetics must be assessed

Lessons learned from osteoporosis and osteoarthritis

Biochemical markers of bone and cartilage turnover are presently the most advanced with respect to matrix remodelling, and some markers may comply with one or more components of the BIPED classification.

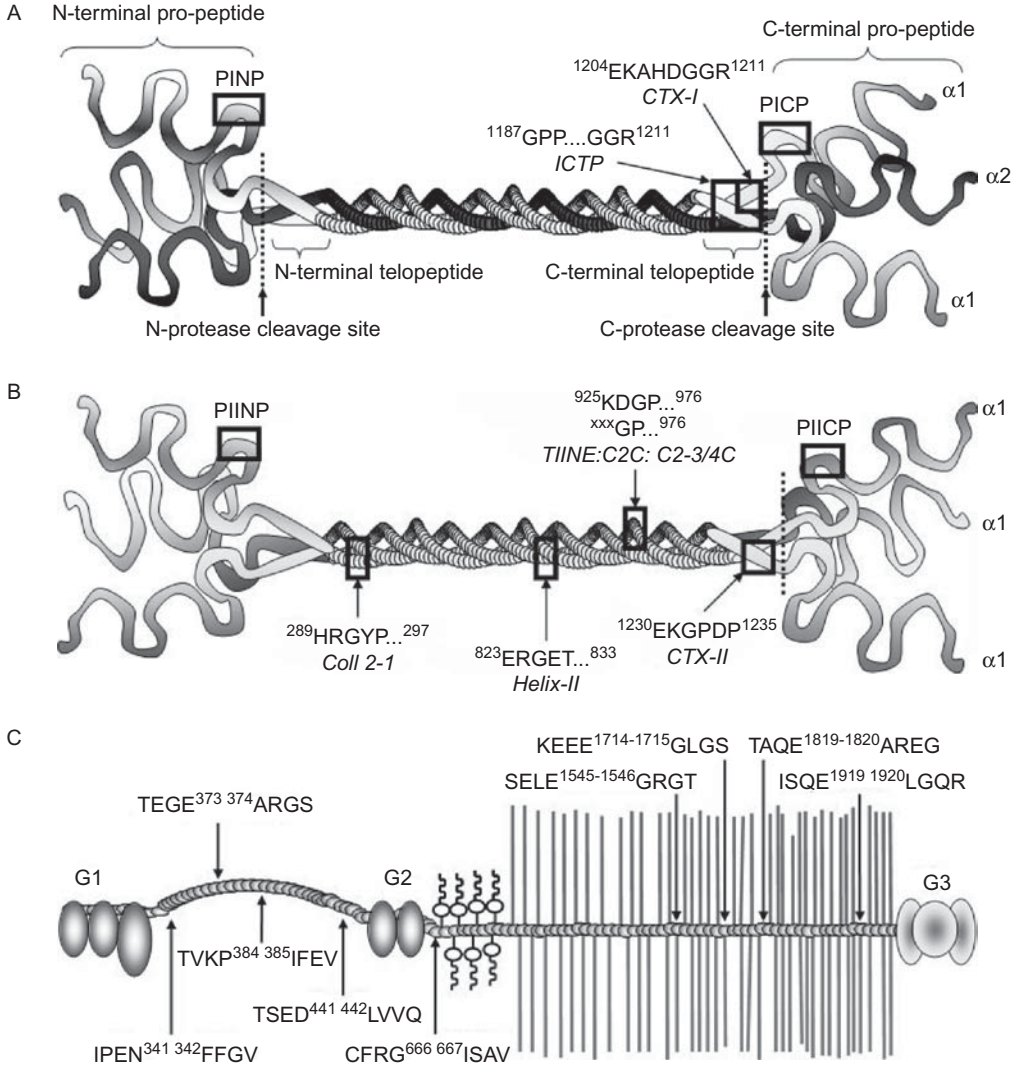

Figure 5. Protease-generated neoepitopes in aggrecan and collagen type I and II. The N- and C-terminal pro-peptides PINP, PICP, PIINP and PIICP in collagen type I (A) and collagen type II (B), respectively, are used to define protein formation, as they are released during formation of the matrix. (A) In contrast, the degradation markers ICTP (MMP-mediated) and C-terminal telopeptide of type I collagen (CTX-I) (cathepsin-K-mediated) located in the C-terminal telopeptide are found in body fluids after degradation of collagen type I. (B) The CTX-II (MMP-mediated) degradation marker is located in the C-terminal telopeptide in collagen type II. Coll 2-1, Helix-II, TIINE, C2C, and C2-3/4C are degradation markers located in the helix of collagen type II. (C) The aggrecan molecule is shown with the MMP cleavage sites (arrow upward) and disintegrin and metalloproteinase with thrombospondin motifs (ADAM-TS) cleavage sites (arrow downward).

However, a long range of biochemical markers measuring degradation, formation and turnover of the bone and cartilage matrices have been developed and applied in translational research (Rousseau et al. 2004a, Rousseau & Delmas 2007). A complete listing and discussion of these important biomarkers that each may have an unique value under specific and different settings are beyond the scope of this present article, but the authors acknowledge many of these important markers that include but not are limited to: TIINE, PIIaP, PIInP, PIIcP, HELIX-II, C2C, C1C2, Coll-2-1, ICRP, 374-ARGS, 342-FFGV, CS846, YKL-40, NTX, COMP, BS-ALP, PINP, CTX-I, CTX-II, osteocalcin, cathepsin K, TRAP (Schaller et al. 2005a, Leeming et al. 2006b, c, Hellio Le Graverand et al. 2006, Nemirovskiy et al. 2007, 2008, Radabaugh et al. 2008, Saxne & Heinegard 1992, Lohmander et al. 1994, Crnkic et al. 2003, Andersson et al. 2006, Dam et al. 2008, Karsdal et al. 2003, 2007c, 2008d, Poole & Dieppe 1994, Bleasel et al. 1999, Fraser et al. 2003, Mullan et al. 2007, Conrozier et al. 2008, Rousseau et al. 2004b, Kondo et al. 2001, Garnero et al. 2002a, Rousseau et al. 2004a, Sumer et al. 2007, Henriksen et al. 2006, 2007b).

The two biochemical markers, C-telopeptide of type I collagen (CTX-I) and CTX-II, are neoepitopes from type I and II collagen, respectively, and are the most widely

used biochemical markers in OP and OA research. The following section describes the use of these markers in translational science and illustrates how the neoepitope approach may be included in a future drug development tool kit.

Collagen type I degradation and bone biology

Bone turnover is a continuous process that ensures calcium homeostasis and bone quality (Seeman & Delmas 2006). Bone turnover is mediated by osteoclasts degrading the bone matrix and osteoblasts forming new bone matrix, two processes which, under normal circumstances, are tightly balanced (Karsdal et al. 2007b).

In addition to the traditional assessment of bone mass by BMD measurement, monitoring of bone turnover – i.e. bone resorption and formation – has led to essential understanding of the pathophysiology of postmenopausal and glucocorticoid-induced OP, Paget's disease, bone metastatic diseases and osteopetrosis, all of which are characterized by pathological changes in bone turnover. These changes often have been demonstrated to be caused by changes in bone resorption.

The osteoclasts promote bone resorption through active secretion of hydrochloric acid, which is mediated by active proton transport and passive chloride transport into the resorption lacuna (Roodman 1999). In an orchestrated sequence of events, proteases are secreted into the resorption lacunae; cathepsin K is the most important of these proteases (Nishi et al. 1999, Hou et al. 1999). Cathepsin K degrades the organic matrix of the bones, in which type I collagen is the most abundant protein (Seeman & Delmas 2006). The degradation of type I collagen by cathepsin K leads to the release of the CTX-I neoepitope (Sassi et al. 2000, Garnero et al. 2003).

In simple *in vitro* cultures of human osteoclasts on bone slices CTX-I release correlates with scoring of pit areas (Figure 6B), as it is released directly from the resorption lacuna below the osteoclasts (Figure 6A) (Henriksen et al. 2004, Schaller et al. 2004). In addition, in *in vitro* resorption experiments the CTX-I release is sensitive to inhibitors of bone resorption and can be used to dynamically monitor resorption inhibition occurring while the culture is ongoing (Sorensen et al. 2007a, b, Henriksen et al. 2004, 2005, Karsdal et al. 2005). In *ex vivo* models of bone resorption, CTX-I release can be used to investigate resorption and has continuously been demonstrated to display sensitivity to both inhibitors of bone resorption and stimulators

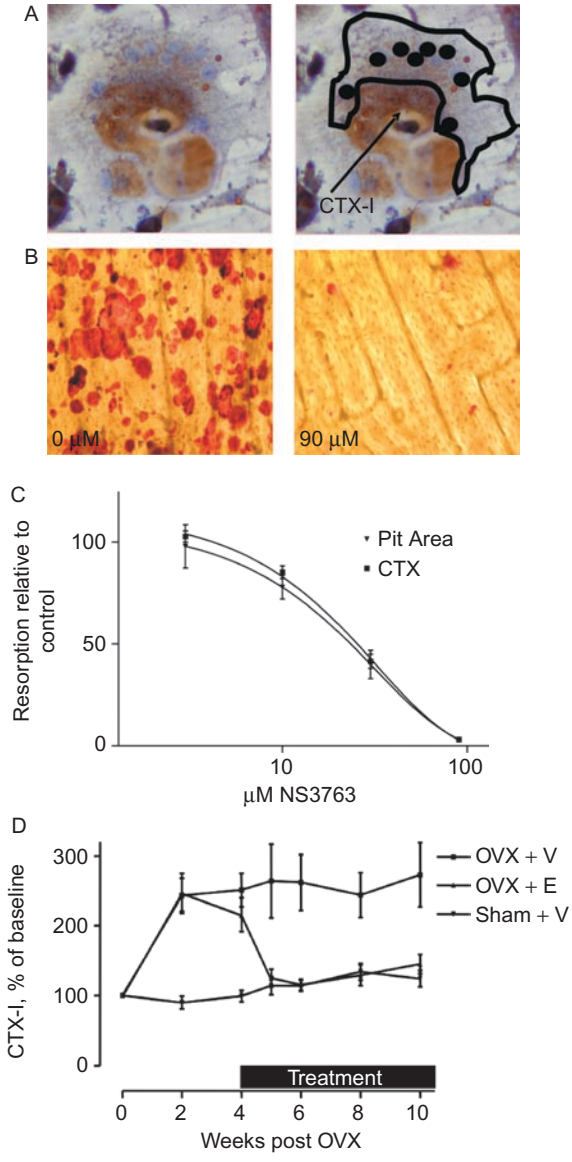

Figure 6. A: Multinucleated osteoclast. CTX-I is located below the osteoclast in the resorption lacunae. B: Pit stainings of the inhibition of bone resorption with the chloride channel inhibitor NS3736. In the presence of 90µM of NS3736, bone resorption is completely abrogated, visualized by the absence of the pits stained in red. Modified from (Schaller et al. 2004). C: Quantification of bone resorption in the presence of the chloride channel inhibitor NS3736. Quantification of pit-formation determined by measurements of CTX-I release in the medium and pit area measurements. These two measurements are in complete alignment, suggesting the validity of CTX-I as a surrogate marker of bone resorption. Modified from (Schaller et al. 2004). D: The suggested animal model for postmenopausal OP in the aged rat ovariectomy model. CTX-I was measured in the serum of fasting animals. After ovariectomy, bone resorption increased relative to that of sham-operated animals; the increase was attenuated by treatment with estrogen. Figure 6 B and C, Reproduced from J Bone Miner Res 2004;19;1144–1153 (Schaller et al. 2004) with permission of the American Society for Bone and Mineral Research.

of bone formation (Garnero et al. 2003, Schaller et al. 2004).

In animal models of bone turnover, such as the FDA-recommended ovariectomized (OVX) rat model for postmenopausal OP, CTX-I increases with the removal of estrogen, corresponding to increased bone resorption, and can be suppressed by estrogen or SERM treatment (Figure 6D) (Hoegh-Andersen et al. 2004, Schaller et al. 2004, Srivastava et al. 2000, Christgau et al. 2004). Additional investigations in the OVX model have demonstrated that reduction in CTX-I in response to antiresorptive treatment leads to improved bone strength (Schaller et al. 2004).

CTX-I levels increase after menopause (Bonde et al. 1995, Rosenquist et al. 1998, Recker et al. 2004). CTX-I can be measured in both urine and serum and, importantly, is sensitive to antiresorptive treatment (Leeming et al. 2006a, Rosenquist et al. 1998, Reginster et al. 2001, Karsdal et al. 2008b), as illustrated in Figure 7A. In patients undergoing antiresorptive treatment, dynamic monitoring of CTX-I during therapy has demonstrated the efficacy of the intervention through correlation with BMD increase (Christgau et al. 1998, Okabe et al. 2004, Kim et al. 2005) (Figure 7A). As a result, CTX-I is being used in a large number of studies (Black et al. 2007, McClung et al. 2006, Black et al. 2006, Ravn et al. 1999a, 2000, 2003, Tanko et al. 2004, Chesnut et al. 2000) as an indicator of bone resorption and as one component in a fracture-risk prediction models. Figure 7A shows that robust evidence of efficacy can be obtained rapidly with this biochemical marker, prior to that of a standard technique such as BMD assessment. Baseline measurement of CTX-I, which correlates with bone turnover rates (Bonde et al. 1995, Christgau et al. 2000, Reginster et al. 2001), has been shown to have prognostic value as an independent predictor of fracture risk. Combined with BMD, age and/or prior fracture rates, it contributes to improvement in the prediction of risk of fracture (Figure 7B).

With respect to assessing bone quality, which is a highly debated topic, biomarkers appear to have a role. Type I collagen undergoes isomerization in the DG motif of the CTX-I epitope (^{1207}EKAHD**DG**R^{1214}) *in vivo* and also *in vitro* (Garnero et al. 2002b, Fledelius et al. 1997, Christgau et al. 2004, Cloos & Christgau 2004). The effect of antiresorptive treatment on the systemic ααCTX/ββCTX ratio is a measure of bone age, which correlates with bone quality (Leeming et al. 2006b, c, Henriksen et al. 2007a, Byrjalsen et al. 2007, Karsdal et al. 2007a). Recently, bisphosphonates were found to induce a significantly higher bone-age profile in comparison with other treatments, such as SERMs, calcitonin and estrogen-replacement therapy, potentially providing insights into bone quality (Byrjalsen et al. 2007, Karsdal et al. 2008a).

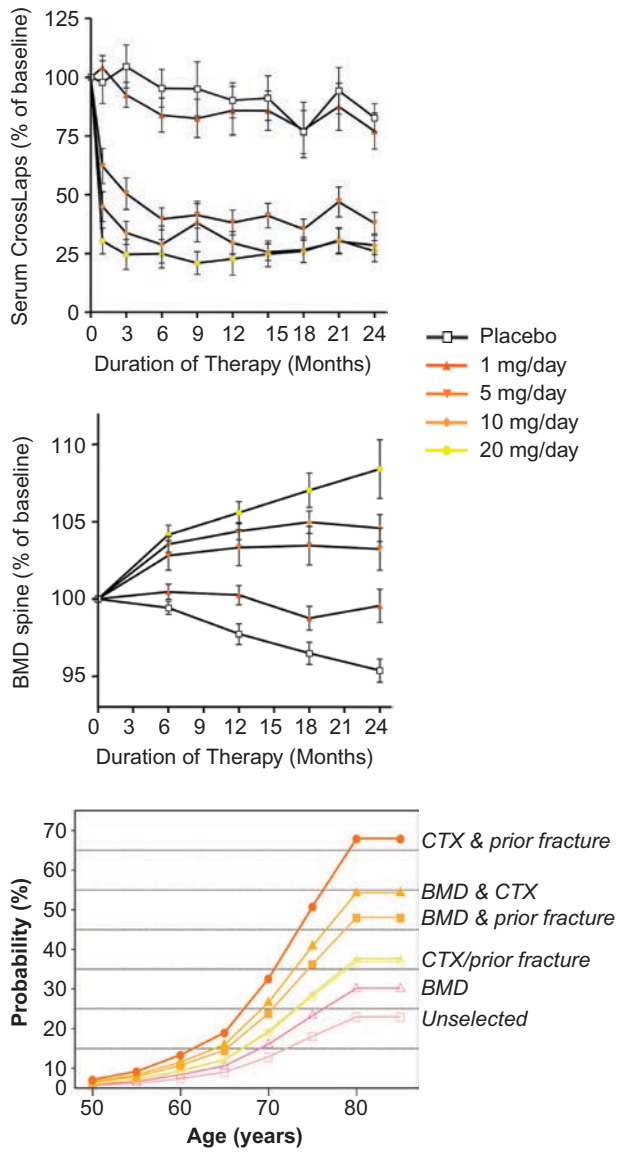

Figure 7. (A, B) Dose-response in biochemical marker CTX-I in response to alendronate in a phase II clinical trial. The sensitivity of response of the biochemical marker CTX-I compared with that of the 'gold standard' BMD provides evidence of efficacy more quickly and in a smaller study population (modified from Ravn et al. 1999a). (C) Illustration of the significant prognostic value of CTX-I alone and in combination with other risk factors for fracture, thus conforming to the BIPED classification. 'CTX and prior fracture' is one combination and 'CTX/prior fracture' means either CTX or prior fracture' (modified from Garnero et al. 1996).

In summary, the cathepsin K-generated collagen type I neoepitope CTX-I is a well-validated, sensitive, easily measured and accurate indicator of bone resorption. As such, it fulfils most of the BIPED criteria and provides proof that neoepitopes generated by a specific combination of enzyme and matrix molecule are highly relevant for monitoring disease risk, progression and response to treatment.

Collagen type II degradation and cartilage biology

OA leads to alterations in the metabolism of the articular cartilage and subchondral bone (Behrens et al. 1989, Stoop et al. 1999, van Meurs et al. 1999, Kerin et al. 2002, Mansell et al. 2007, Karsdal et al. 2008c). Cartilage is for the most part composed of collagen type II (60–70% of dry weight) and proteoglycans (10% of dry weight), of which aggrecan is the most abundant (Kiani et al. 2002). Cartilage degradation is mainly mediated by the MMPs and the closely related ADAM-TS (a disintegrin and metalloproteinase with thrombospondin motifs) (Little et al. 2005a, b, Stanton et al. 2005, Collins-Racie et al. 2004, Mort et al. 2003, Tang 2001, Koshy et al. 2002, Dean et al. 1989, Martel-Pelletier et al. 1999, Reboul et al. 1996, Pelletier et al. 2004, Helminen et al. 2002, Van den Berg 2002). Aggrecan is degraded by both MMPs and ADAM-TS, whereas collagen type II is degraded principally by various MMPs (Hui et al. 2003, Little et al. 2005a, b, Stanton et al. 2005, Collins-Racie et al. 2004, Mort et al. 2003, Tang 2001). The action of these proteases results in the release of various extracellular fragments that can be measured both *in vitro* and *in vivo* (Schaller et al. 2005a) and can be used as a measure of specific protease-mediated cartilage degradation.

Since type II collagen is the most abundant protein in cartilage, several different degradation fragments of collagen type II have been indicated as useful for monitoring degenerative diseases of the cartilage (Schaller et al. 2005a, Sumer et al. 2006). CTX-II is an MMP-generated neoepitope derived from the C-terminal part of type II collagen (Christgau et al. 2001, Mouritzen et al. 2003), and measurement of CTX-II is very useful for monitoring degradation of type II collagen in experimental set-ups assessing cartilage degradation (Schaller et al. 2005a, Christgau et al. 2001, Mouritzen et al. 2003).

Ex vivo cultured explants of bovine articular cartilage constitute a highly useful model for studying cartilage degradation and formation (Sondergaard et al. 2006a, b, Sumer et al. 2007, Olsen et al. 2007, Karsdal et al. 2007c). In this model, marked increases in cartilage degradation can be induced by the combination of tumour necrosis factor (TNF)-α and oncostatin M (OSM), which time- and dose-dependently induce cartilage degradation as demonstrated by CTX-II (Figure 8A, B). Furthermore, illustrating the role of MMPs in cartilage degradation, CTX-II release was completely abrogated by the addition of the MMP inhibitor GM6001, but not the cysteine proteinase inhibitor E64. E64 actually augmented CTX-II release, probably because of compensation by MMPs (Figure 8C). Immunohistochemical localization of CTX-II reveals that it is highly present in TNF-α- and OSM-treated explants, in areas corresponding to proteoglycan depletion, whereas the MMP inhibitor-treated explants showed no CTX-II (Figure 8D) (Sondergaard et al. 2006a). Biochemical studies of MMP-mediated type II collagen degradation showed that both MMP-9 and MMP-13 possessed the ability to generate the CTX-II fragment (Sondergaard et al. 2006a). In *in vivo* models of OA, such as ovariectomized rats, CTX-II levels have been correlated to erosion of the articular cartilage in rat knees (Hoegh-Andersen et al. 2004), and furthermore, the CTX-II levels respond to estrogen as well as SERM treatment (Hoegh-Andersen et al. 2004, Christgau et al. 2004). Additional analysis of CTX-II demonstrated that it was localized in the eroded areas of the articular cartilage (Oestergaard et al. 2006a, b, c), and investigation of extracted knees from rats that had undergone anterior cruciate ligament trans-section demonstrated that CTX-II was markedly elevated in the operated knee compared with the sham knee after 14 days (Nielsen et al. 2007). These combined findings suggest the relevance of CTX-II as a marker of cartilage degradation and pathology.

In clinical studies, high levels of CTX-II were shown to be associated with the diagnosis of OA (Figure 9A) and to have prognostic value for OA progression (Figure 9B) (Reijman et al. 2004). Although currently there is no treatment for OA, clinical trials assessing reduction in cartilage turnover mediated by different types of treatments are ongoing. Calcitonin, which appears to possess chondroprotective effects, has been shown to reduce CTX-II levels as well as decrease the functional disability caused by the OA (Bagger et al. 2005, Manicourt et al. 2006).

Thus, the MMP-generated collagen type II neoepitope CTX-II is a well-validated, sensitive and accurate indicator for cartilage and fulfils most of the BIPED criteria, although its responsiveness to treatment continues to undergo intensive investigation. Furthermore, CTX-II provides additional proof that neoepitopes generated by a specific combination of enzyme and matrix molecule are highly relevant for monitoring disease risk and progression, as well as response to treatment.

Biomarkers of MRI in osteoarthritis

Better assessment of disease may be achieved through a combination of markers, preferably of different origin, such as imaging studies, which are static, and biochemical markers, which are dynamic (Figure 2). The progression of OA is heterogeneous and can differ significantly between individuals. However, some of the central biochemical changes observed in a population of patients with OA may be more homogeneous. During very early OA, such central changes are proteoglycan degradation and disruption of the collagen matrix (Stanton et al. 2005, Sondergaard et al. 2006a, Glasson et al. 2005) followed by

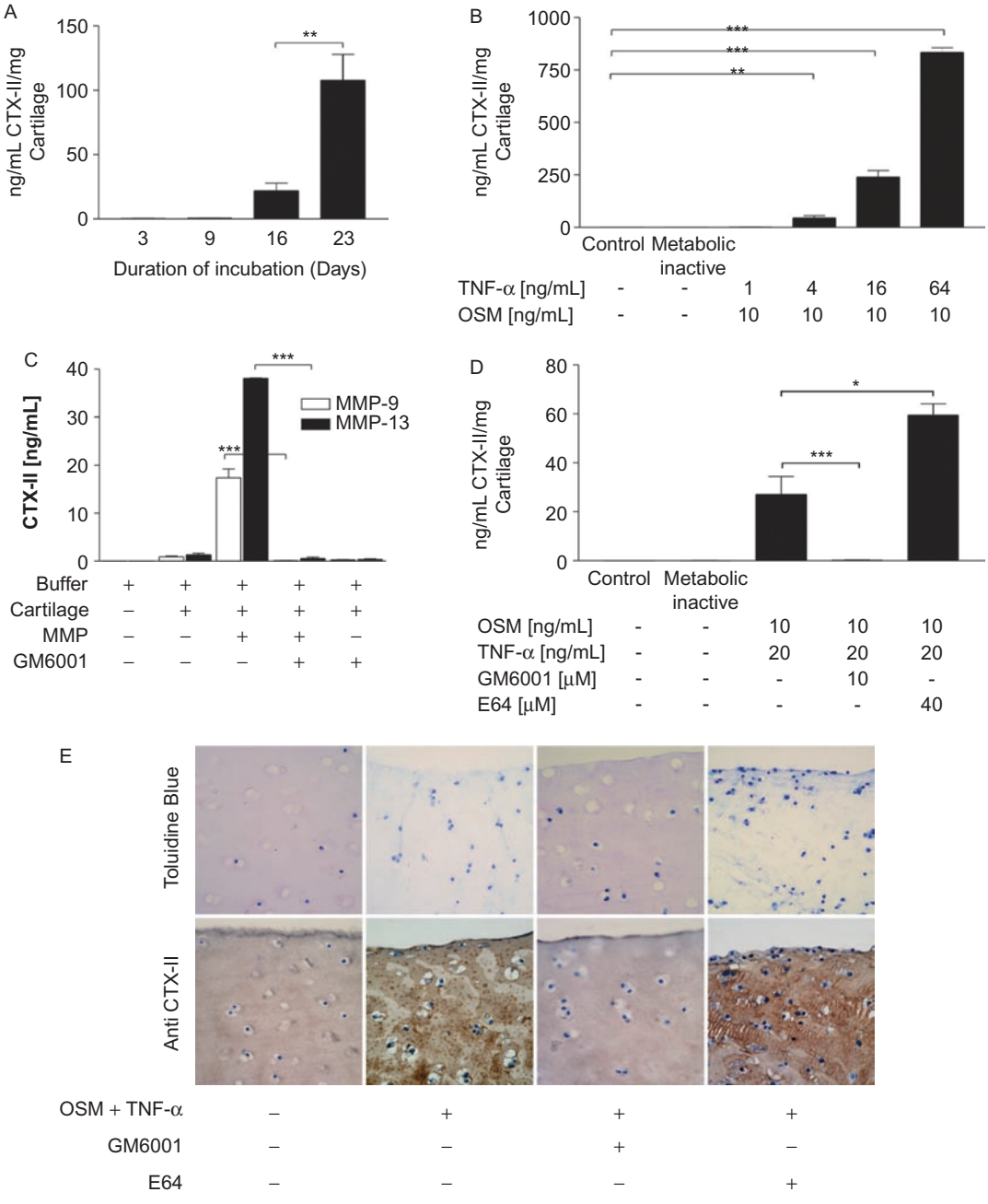

Figure 8. OSM and TNF-α dose- and time-dependently induce cartilage degradation monitored by CTX-II release. (A) CTX-II release as a function of time. (B) CTX-II released as a function of cytokine concentration. (C) CTX-II is generated by MMPs, in this case MMP-9 and MMP-13. (D) Inhibition of the MMPs, but not cysteine proteinases, abrogates CTX-II release from cytokine-stimulated cartilage explants. (E) Immunohistochemical analysis of the presence of CTX-II in explants exposed to cytokine stimulation in the presence or absence of protease inhibitors. Modified from Sondergaard et al. (2006a).

increased water concentration and local oedema. These changes affect the internal cartilage structure only and are probably not observable in terms of cartilage quantity, but are accompanied by increased turnover in cartilage and bone. Following these biochemical changes, there will potentially be a gradual loss of cartilage surface integrity, resulting in early fibrillation. In parallel, effects linked to early radiographic OA may begin in terms of osteophytic lipping. At this relatively progressed stage of OA, focal subchondral bone thickening and focal cartilage loss are likely to occur. Late-stage OA eventually leads to severe cartilage loss and complete denudation. At this stage, the loss of mobility due to joint remodelling and pain may mean joint replacement surgery is the only feasible intervention. This chain of events is illustrated in the Figure 10.

The proposed model is probably too simplified to apply to the progression of OA in the individual, but it may be appropriate for clinical studies in which the focus is on the typical events occurring in the population as a whole. The Figure shows that the current 'gold standard' for defining the degree of OA (the KL index, defined from radiographs (Kellgren & Lawrence 1957)) is focused on the later stages of OA, when osteophytes and cartilage loss are prominent. In addition, JSN by itself is also probably most prominent in the relatively late stages of the disease. Thus, the use of KL and JSN in clinical trials may implicitly favour intervention strategies directed at late-stage OA. For treatments targeting earlier stages of OA, markers relevant to the early stages of disease are needed for efficacy validation. The biochemical markers of wcartilage and bone turnover discussed above are candidates for such early-stage markers.

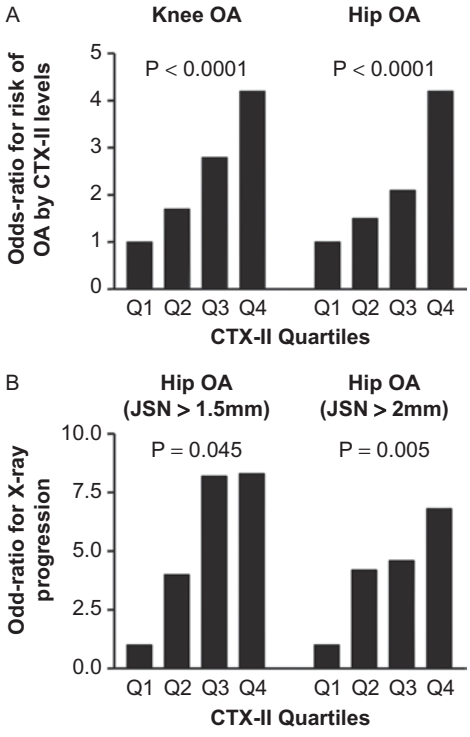

Figure 9. High CTX-II levels in urine were significantly associated with an increased odds ratio of having OA (diagnosis) (A) and to predict progression of OA (B). Modified from Reijman et al. (2004).

In recent years, markers based on imaging have been the subject of much research. We have developed a range of cartilage biomarkers based on fully automated, computer-based analysis of MRI, corresponding to the described chain of events. The biomechanical changes are targeted by a marker of cartilage curvature (Folkesson et al. 2008), the internal structure and surface structure are quantified by homogeneity (Qazi et al. 2007) and smoothness (Folkesson et al. 2008) measures, and the cartilage loss is assessed by thickness (Dam et al. 2007a) and volume quantifications (Folkesson et al. 2007). Such MRI-based markers can supplement biochemical markers because they are fundamentally complementary. The MRI markers are specific to an anatomical site (e.g. a subregion of a cartilage compartment) and they typically quantify the current status (e.g. cartilage volume), whereas the biochemical markers are more focused on the current rate of change (e.g. cartilage turnover). This complementary nature of biochemical and MRI markers suggests that they can be combined to form superior aggregate markers. It was reported recently that while both a biochemical marker of cartilage breakdown (CTX-II) and an MRI marker of cartilage structure (homogeneity) are strongly predictive of early radiographic progression of OA (OR, 6.7 and 4.9, respectively), the combination of these markers with cartilage volume, area, and surface smoothness was far superior (Dam et al. 2007b). The aggregate marker, denoting cartilage longevity, had an odds ratio (OR) of 14.6.

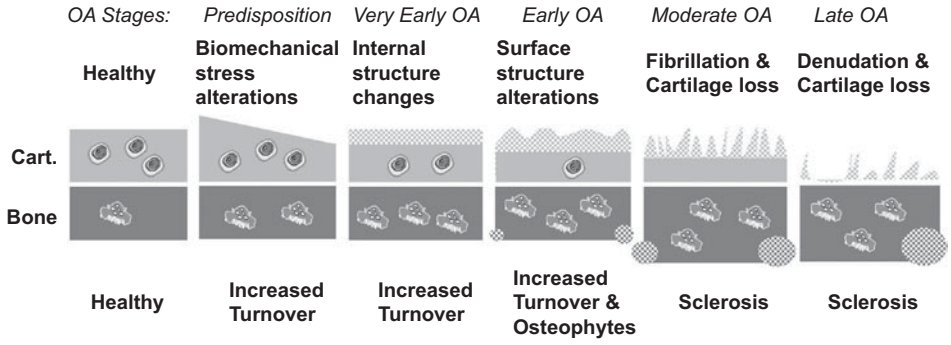

Figure 10. Iconic illustration of the principal stages of OA progression. Despite the heterogeneous aetiology of OA, there may be some common changes in cartilage and bone that are typical for a population. In general, biomechanical and biochemical changes occur before cartilage denudation and subchondral thickening. The effects are illustrated at the stage they are initiated (e.g. a biomechanical imbalance is expected to persist and possibly worsen during the following stages). The bone icons symbolize osteoclasts and osteophytes, and the cartilage icons illustrate chondrocytes. Modified from Qvist et al. (2008).

The superiority of a combination of markers targeting biochemical turnover as well as cartilage quantity and quality (measured from MRI) to any of the individual markers alone indicates that a reductionistic approach is too narrow to capture the complex nature of OA.

The future

We have described how biochemical markers may assist drug discovery and development in the context of the recent publication of the FDA Critical Path and the classification of biomarkers according to the BIPED criteria. Biomarkers are emerging as valid tools in drug development. Implementation of biomarkers in research and development and in the interface with patients may result in significant achievements. In the following section, we highlight some of the myriad potential uses of validated biochemical markers.

Labelling extension

The potential use of calcitonin in OA is an example of a possible label extension fostered by biomarkers. In a post hoc assessment of a phase II clinical trial of oral calcitonin in OP, biochemical markers of cartilage degradation demonstrated that calcitonin attenuated this process thus identifying calcitonin as a potential treatment for OA (Bagger et al. 2005, Tanko et al. 2004). This is an interesting example of the way material from clinical trials, for example, may be reanalyzed using biochemical markers, potentially leading to the identification of novel disease indications and prompting labelling-extension programmes.

Design of proof of concept studies in osteoarthritis

Presently there are no structure-modifying drugs available for the treatment of OA (Qvist et al. 2008). Several clinical studies have been performed testing potential disease-modifying activity (Spector et al. 2005, Bingham et al. 2006, Buckland-Wright et al. 2007, Krzeski et al. 2007) but all have failed to demonstrate such an effect. This may be due to problems with clinical trial design in OA, resulting in part from inadequate understanding of the disease and lack of understanding of disease progression (Murphy & Nagase 2008). However, important lessons have been learned, from these studies, namely that some biochemical markers were able to predict in which patients the disease would progress (Garnero et al. 2008). These markers may be useful in future clinical trials.

The lack of success in achieving disease modification has resulted in the pharmaceutical industry being more hesitant to venture into large-scale phase III clinical studies in a disease that is both poorly understood and studied in clinical trials. This may be an ideal opportunity for biomarker use and developments, as these ultimately should boost confidence, reduce costs of conducting clinical trials and lead to more drug development strategies being tested in OA. A combination of several different biochemical markers reflecting different disease parameters in combination with advanced MRI techniques, may in the future be used for the design of smaller proof of concept studies (Karsdal et al. 2008c, Dam et al. 2008).

Identification of competitive advantages by increased efficacy – uncoupling of bone resorption and bone formation

In OP, there is an increase in both bone resorption and bone formation, albeit with a larger increase in bone resorption, which leads to a continuous loss of bone. Bisphosphonate, estrogen and SERM treatment results in a decrease in both bone resorption and bone formation, limiting efficacy (Karsdal et al. 2007b, 2008e). However, some treatments may decrease bone resorption without affecting bone formation and may be identified by validated biochemical markers of bone turnover (Karsdal et al. 2007b). Use of biochemical markers of turnover in preclinical settings, combined with analysis of patients with mutations in osteoclasts attenuating acidification of the osteoclastic resorption lacunae, has identified inhibition of bone resorption with positive effects on bone formation (Karsdal et al. 2005, 2007b, 2008e, Henriksen et al. 2004, 2007b, Sorensen et al. 2007c, Schaller et al. 2004, 2005b). This example suggests that biochemical markers may aid the detection of novel modes of action leading to increased efficacy. Another example is the current development of oral calcitonin. Oral calcitonin reduces bone resorption but does not result in a secondary effect on bone formation (Tanko et al. 2004).

Safety

Biomarkers identified for and used as efficacy markers in one disease might be applied to another disease as safety markers. By using these markers in both preclinical and clinical research, identification of target-specific effects may be improved. An example involves the highly debated insulin sensitizers belonging to the thiazolidinedione class (rosiglitazone, pioglitazone). These agents have been approved by the FDA, although they have been demonstrated to have detrimental effects on bone health (Meier et al. 2008, Meymeh & Wooltorton 2007, Berberoglu et al. 2007, Short 2007). Of great concern is that this detrimental effect might easily have been recognized in preclinical development and in clinical trials through the use of a biochemical marker of bone

formation, such as osteocalcin or bone-specific alkaline phosphatase activity (Meier et al. 2008, Meymeh & Wooltorton 2007, Berberoglu et al. 2007, Short 2007).

By implementing validated biochemical markers of bone turnover in preclinical toxicology studies, and in phase II, or even shorter Phase IIA proof of concept studies, detrimental effects on bone or cartilage for treatments developed for other indications may be identified and dealt with diligently. It may even be suggested that relatively inexpensive tools such as biochemical markers may in the future be implemented in standard safety packages in both preclinical and clinical testing.

Profiling screening

Some evidence suggests that the traditional, reductionistic approach of targeting single enzymes may be supplemented by a whole-tissue-pathology approach. An example involves cAMP modulation of chondrocytes.

Calcitonin is a physiological modulator of osteoclast function and a well-established antiresorptive medication, which has long been used for the treatment of OP (Cranney et al. 2002). Studies have shown that binding of calcitonin to its receptor activates the cAMP–protein kinase A and the Ca^{2+}–protein kinase C signalling pathways (Sexton et al. 1999, Purdue et al. 2002, Hilton et al. 2000). We recently demonstrated a pharmacological effect of calcitonin on chondrocytes, in part mediated by increased cAMP levels, resulting in a general decrease in MMP activity (Olsen et al. 2007).

We implemented a whole-tissue-pathology approach by culturing whole articular cartilage explants in the presence of catabolic induction cytokines. Using the well-defined neoepitopes of cartilage degradation – MMP-mediated collagen type II degradation (CTX-II) and MMP-mediated aggrecan degradation (FFGV-G2) – and the aggrecanase-mediated aggrecan assay (ARGS-G2), we found that stimulators of the adenylate cyclase enzyme and inhibitors of phosphodiesterases influenced degradation of both collagen type II and aggrecan; this degradation was mediated though a dose-dependent synthesis and activation of MMPs (Karsdal et al. 2007c). These data demonstrate that cAMP levels in chondrocytes are an important indicator of the phenotype of the chondrocytes and, interestingly, reveal a target that would have been missed by the traditional reductionistic approach of focusing on the proteases responsible for the degradation of cartilage itself.

The approach of using a large panel of biochemical markers of tissue degradation and tissue formation in the screening of potential therapeutic compounds may better take into account whole-tissue pathophysiology, making it possible to identify drugs based on a mode of action that would not otherwise have been recognized.

Personalized medicine

In the interaction between physicians and patients, in which patients are demanding more information and proof of efficacy than could have been provided previously on the basis of traditional research techniques, biomarkers of different types may be of increased assistance. Use of a combination of imaging and biochemical techniques to profile the individual patient may aid in the selection of the optimal treatment. In the musculoskeletal field, it has been demonstrated that patients with high rates of bone turnover gain more bone than those with low bone-turnover rates when treated with antiresorptive therapies (Civitelli et al. 1988, Rosen et al. 1997, Greenspan et al. 1998, Sarkar et al. 2004). This is an interesting example of the way a combination of diagnostic and prognostic markers may be used to identify patients for whom a particular treatment would be optimal. In addition, use of markers of efficacy for monitoring purposes may improve patients' motivation to adhere to a long-term treatment. Such markers may allow for early recognition of non-responders to treatment, facilitating a timely switch to another therapy.

In the future, the development of several biochemical and structural biomarkers of prognosis in OA may aid the identification of those patients who will benefit the most from treatment (Dam et al. 2008, Qvist et al. 2008). If a panel of biomarkers were to be used, these may aid in the selection of the treatment for the individual person. With respect to the pathogenesis of OA, there are different substages of the disease with either more cartilage or bone involvement (Qvist et al. 2008, Karsdal et al. 2008c, d), as well as reversible and irreversible processes (Qvist et al. 2008, Karsdal et al. 2008d). Thus in the selection of the most optimal treatment, the identification of the specific stage of the disease is likely to benefit patients. One treatment that may be proven efficacious at one stage of the disease may fail at yet other stages of the diseases (Qvist et al. 2008). As such, this should be considered when designing intervention strategies for individual patients.

May neoepitopes be a viable biomarker approach for other pathologies?

Many pathologies are tissues specific and involve ECM turnover and changes. Matrix remodelling is an integrated process of tissue development, maintenance and pathogenesis. During the development of most pathologies, the composition of the ECM changes. The key constituents of ECM of many tissues are collagens and proteoglycans, each with their own unique biophysical properties. Endopeptidases such as MMPs and cysteine proteases play major roles in the degradation of extracellular macromolecules such as the collagen and proteoglycans.

Thus optimal neoepitope programmes may be designed for many different diseases by identification of the major pathological proteases in combination with the predominant extracellular proteins in that tissue. If the neoepitope design is approached most optimally, the given disease is carefully investigated for the expression and identification of different proteases and proteins at different stages during progression of the pathology. This will allow both biomarkers that will comply with the BIPED criteria but in addition will enable staging of diseases.

For example, in atherosclerosis one of the main proteases gaining attention has been MMP-9, secreted by activated macrophage foam cells and eventually leading to destabilization of the atherosclerotic plaque followed by rupture. Collagen type III is the major specific collagen of the arterial wall. Possibly in the future, MMP-9 degraded collagen type III may prove to have a similar utility as CTX-I and CTX-II.

Conclusion

As the cost of drug development continues to increase and the number of approved drugs continues to decrease, reanalysis of the drug development roadmap and technical tools seems a prerequisite for improvement.

We have advocated for further development and implementation of dynamic biochemical markers of tissue turnover, in combination with static assessments of tissue status, to ensure successful progress along the drug development critical path from *in vitro* studies to preclinical experiments and, finally, to clinical trials. By incorporating biochemical markers in all aspects of drug discovery and development, novel treatment opportunities may be identified, research effort may be stimulated by the promise of efficacy, and toxicity may be identified early.

Combined use of advanced techniques may aid in the recognition of projects with the highest chance of success at early decision points on the development path, ensuring best use of limited resources

An optimal tool kit is needed, and biochemical markers based on the neoepitope approach may be one important tool to be used in combination with others. Importantly, these markers must enable translational science to transfer information gained from preclinical evidence to clinical practice, and to transfer knowledge of human pathology to preclinical models of disease and therapeutic efficacy.

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