Biomarkers, 2011; 16(3): 193–205

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2011.557440

informa
healthcare

COMMENTARY

Post-translational modifications of the extracellular matrix are key events in cancer progression: Opportunities for biochemical marker development

D. J. Leeming, A. C. Bay-Jensen, E. Vassiliadis, M. R. Larsen, K. Henriksen, and M.A. Karsdal

Nordic Bioscience A/S, Herlev, Denmark

Abstract

The aim of this review is to discuss the potential usefulness of a novel class of biochemical markers, designated neoepitopes. Neoepitopes are post-translational modifications (PTMs) of proteins and are derived by processes, such as protease cleavage, citrullination, nitrosylation, glycosylation and isomerization. Each PTM results from a specific local physiological or pathobiologial process. Identification of each modification to a tissue-specific protein may reveal a unique disease-specific biochemical marker. During cancer metastasis, the host tissue is extensively degraded and replaced by cancer-associated extracellular matrix (ECM) proteins. Furthermore, severe cellular stress and inflammation, caused by cancer, results in generation of PTMs, which will be distributed throughout the ECM. This gives rise to release of protein-specific fragments to the circulation. Here we highlight the importance of remodeling of the ECM in cancer and the generation of PTMs, which may be cancer specific and reflect disease progression; thus having potential for biochemical marker development.

Keywords: Neoepitope, biochemical marker, FDA, critical path, translational science, biomarker, cancer

Introduction

The ability of a tumor to successfully metastasize to a new location depends largely on the composition of the extracellular matrix (ECM) of the tissue in which a tumor is growing or the site of metastasis. Mintz and colleagues showed that the normal mouse embryonic tissue microenvironment could repress expression of the tumor phenotype (Mintz & Illmensee, 1975; Dolberg & Bissell, 1984). To maintain healthy tissue, the ECM must regenerate itself by normal remodeling. Old or damaged proteins are broken down in a specific sequence of proteolytic events and replaced by new proteins. However, during pathological conditions, such as cancer, fibrosis and inflammation, the delicate repair-response balance is disturbed (Schuppan et al. 2001; Ingber, 2008). The original proteins of the ECM are replaced by different matrix constituents and consequently the composition and quality of the matrix is altered. During cancer propagation, the ECM may be stiffened and this can actually enhance tumor cell migration, which has been observed using intravital imaging along type I collagen fibers adjacent to invading breast cancer cells (Condeelis & Pollard, 2006; Ingman et al. 2006).

During pathological remodeling of the ECM, excessive levels of tissue- and pathology-specific turnover products are released into the circulation and these may be targeted as biochemical markers. Turnover products holding these PTM modifications are released into the circulation during progression of cancer, and are referred to as neoepitopes. Neoepitopes are a special class of post-translational modifications (PTMs) and are defined as modifications made secondary to translation of the protein into the peptide sequence from mRNA. Thus, most PTMs are not DNA coded, but are rather a consequence of tissue physiology and pathophysiology (Cloos & Jensen, 2000; Cloos & Christgau, 2004). PTMs may be derived from processes, such as aging (amino acid isomerization), citrullination, protease degradation and glycosylation (Cloos & Jensen, 2000; Cloos & Christgau, 2004). Protease-generated neoepitopes

Address for Correspondence: Karsdal M. A., Nordic Bioscience A/S, Herlev Hovedgade 207, DK-2730 Herlev, Denmark. Phone: + 45 44 52 52 10. Fax: + 45 44 52 52 51. E-mail: MK@nordicbioscience.com

(Received 11 November 2010; revised 20 January 2011; accepted 20 January 2011)

Abbreviations

ADAMTS	a disintegrin and metalloproteinase with a thrombospondin type 1 motif
AGE	advanced glycation/glycosylation endproduct
BIPED	burden, investigatory, prognostic, efficacy and diagnostic
BMD	bone mass density
CCP	cyclic citrullinated peptide
CRP	C-reactive protein
CTX-I	C-terminal telopeptide of type I collagen
CTX-II	C-terminal telopeptide of type II collagen
CVD	cardiovascular diseases
DPD	deoxypyridinoline
ECM	extracellular matrix
ECMR	ECM remodeling
JSW	joint space width
MMP	metalloproteinase
OA	osteoarthritis
OP	osteoporosis
PIIANP	type IIA procollagen N-terminal peptide
PADs	peptidylarginine deiminases
PTM	post-translational modifications
PYD	pyridinoline
RA	Rheumatoid arthritis
ROS	reactive oxygen species
SERM	selective estrogen receptor modulator

have, to date, received more attention than other PTMs. However, potentially important PTMs that are believed to be specific for cancer and other pathological conditions have recently been identified (Cloos & Christgau, 2004; Karsdal et al. 2010).

Recent studies suggest that PTMs may not only be useful biomarkers for early detection of malignant tumors, but they may also contribute to abnormal cellular proliferation, adhesion characteristics and morphology (Krueger & Srivastava, 2006) and may cause many of the differences between normal and cancer tissue (Marx, 2004; Bosques et al. 2006; Krueger & Srivastava, 2006; Spickett et al. 2006; Hanash et al. 2008; Sawyers, 2008). Thus, PTM profiles may be used as "biochemical footprints" for detecting and verifying the function and activity of key cellular signaling pathways(Marx, 2004; Bosques et al. 2006; Krueger & Srivastava, 2006; Spickett et al. 2006; Hanash et al. 2008; Sawyers, 2008).

The aim of this review is twofold. First, it highlights the importance of the ECM for controlling cell fate. Second, it will investigate the PTMs applied to the ECM during cancer invasion as these may serve as a target for biochemical marker development.

Function of the ECM

The ECM is a three-dimensional structure that encapsulates the cells and define the cellular microenvironment (Aumailley & Gayraud, 1998). It consists of a meshwork of proteins to which soluble factors, such as growth factors and cytokines, can bind. There are two main types of ECM. The first is the basement membrane (BM), which interacts directly with the epithelium and endothelium, and it is composed of primarily of collagen IV, laminins, entactin/nidogen and heparan sulfate proteoglycans (e.g., perlecan; Figure 1; Yurchenco & Schittny, 1990).

The second type is the interstitial matrix, which makes up the bulk of the ECM in the body. The interstitial matrix consists of many collagens including types I and III, which together with fibronectin contribute to the mechanical strength of the tissue (Kozaci et al. 1997; Li et al. 2004). The interstitial matrix additionally consists of tenascin and proteoglycans that provide tissue hydration, enable binding of growth factors and cytokines to the tissue, and cross-link the matrix to enhance its integrity (Bosman & Stamenkovic, 2003).

Although originally considered as merely a support system for the cells within the tissue, the ECM is now recognized as a central regulator of cell and tissue behavior via transmembrane signaling (Dolberg & Bissell, 1984; Bissell & Aggeler, 1987; Lochter & Bissell, 1999; Bissell & Radisky, 2001; Radisky & Bissell, 2004). While the basic characteristics and composition of the BM and interstitial matrix are constant across tissues, variations in ECM components, such as protein isoform expression and PTMs, contribute to differences in ECM organization and structure and ensure tissue specificity (Cloos & Christgau, 2004). Matrix components and PTMs, such as glycosylation and cross-linking, significantly affect the mechanical properties of the ECM, including its viscoelasticity or stiffness. Both the stiffness and topology (three-dimensional appearance) of the ECM regulate the

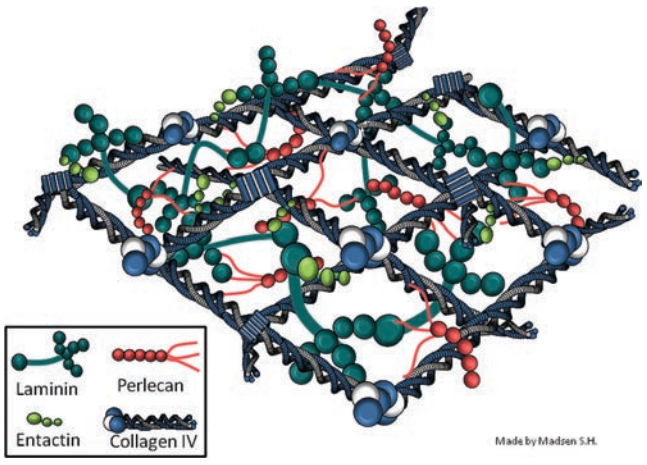

Figure 1. The molecular structure of a typical basal lamina. The basal lamina (A) is formed by specific interactions between the proteins type IV collagen, laminin, and entactin plus the proteoglycan perlecan (B). Arrows in (B) connect molecules that can bind directly to each other. Figure 1 drawn by Suzi Høgh Madsen, Nordic Bioscience.

growth, remodeling, differentiation, migration and phenotype of a wide variety of cell and tissue types (Paszek et al. 2005).

ECM in cancer-related upregulation of tissue turnover

The ECM not only maintains the three-dimensional structure of tissues and organs, but also plays critical roles in cell proliferation, differentiation, survival and motility. The architecture of tumor-associated ECM is fundamentally different from that of the normal tissue stroma (Clarijs et al. 2003). As an example, type I collagen is situated parallel to the epithelial cells in healthy tissue, but is less organized in the stroma surrounding a metastasized tumor (Ruiter et al. 2002). These changes to the ECM of the stroma promote transformation, tumor growth, motility and invasion, enhance cancer cell survival, enable metastatic dissemination, and facilitate the establishment of tumor cells at distant sites (Ruiter et al. 2002). Matrix degradation can additionally promote malignant progression and metastasis.

ECM components and remodeling enzymes are elevated in the circulation in cancer patients (Yu et al. 1997; Winding et al. 2002). Cancer is a disease caused by the disregard of essential rules governing how cells should organize in a stable manner within the tissue of all living beings. Uncontrolled cell growth is necessary for cancer formation. Such growth becomes self-directed, leading to a disorganization of the normal tissue architecture which is known as "neoplastic transformation." More than 90% of malignant tumors are epithelial tumors (Ingber, 2008), occurring where there is a collapse in the boundary between the epithelial and connective tissues that encompass a given organ. Interruption of these tissue boundaries enables cancer cells to enter nearby blood vessels or the lymph node system, thus spreading or "metastasizing" to remote organs resulting in multiorgan failure and death. These processes affect the ECM as well as the proteases, cells and proteins found in the ECM. As illustrated in Figure 2, cancer cell metastasis results in extensive ECM remodeling resulting in the release of matrix components, neoepitopes, into the circulation.

Proteases

Metalloproteinases (MMPs)

MMPs are a large family of proteases which include the stromelysins (MMP-3 and -10), collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), matrilysins (MMP-7 and -26) and the membrane-type MMPs (MMP-14, -15, -16, -17, -24 and-25; Roycik et al. 2009). These MMPs are able to degrade almost all components of the BM and ECM. In tumors, the stringent control of MMP expression and activity is lost (Strongin, 2006), resulting in extensive overexpression of a range of MMPs (Lochter et al. 1997). MMPs are involved in cancer progression by cleavage of the ECM, thus releasing several molecules embedded in the ECM. The released molecules can inhibit apoptosis and enable cell invasion into the tissue (Sternlicht & Werb, 2001). MMPs also support angiogenesis and alter immune responses, blocking immune surveillance, with the overall effect of stimulating tumor growth (Overall & Lopez-Otin, 2002). The tissue inhibitors of metalloproteinases (TIMPs) either directly inhibit the activity of MMPs by forming tight, noncovalent inhibitory complexes with them, or control the activation process itself (Duffy, 1996). A tight equilibrium between MMPs and TIMPs is essential for normal tissue function; however during cancer growth and metastasis, this is disrupted. It is not only through degradation of the ECM that invasion of cancer cells is promoted, also ECM degradation itself results in the release of embedded growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which stimulate angiogenesis and tumor growth. These growth factors are also implicated in the synthesis and release of collagenases and in the

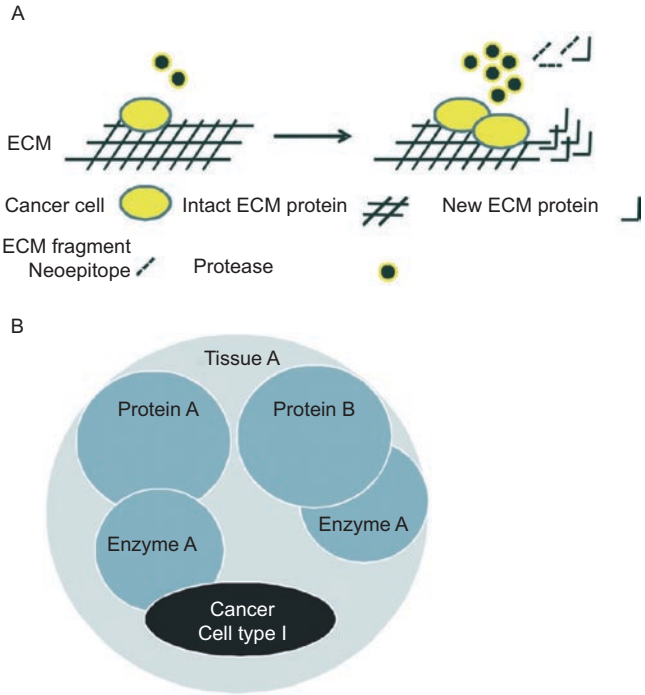

Figure 2. Schematic representation of the generation of neoepitope markers. (A) Cancer cells invade the matrix by expression of a battery of proteolytic enzymes. These enzymes degrade the ECM, releasing smaller fragments of protein from the ECM into the circulation. In addition the cancer cell produce a range of proteins that are sequestered in the matrix (B) Schematic representation of the design and origin of an optimal biomarker. The overlapping area in the circles represents the common denominator of biomarkers, which is needed to obtain tissue specificity and sensitivity. Different cancer cells predominantly express given proteases, while different tissues contain signature proteins. By carefully examining these relationships, a biomarker may be designed, for example, a cancer cell expressing MMP-9, metastasizing to a tissue with signature proteins, that is, basement matrix with type IV collagen. Thus, an MMP-9 fragment of type IV collagen may provide a possible biochemical marker of the initiation and progression of that given cancer cell type in that tissue.

additional release of the urokinase form of plasminogen activator (uPA) by the endothelial cells of blood vessels surrounding the tumor, thus upregulating both the proteolytic flow and angiogenesis (Blood & Zetter, 1990).

MMPs related to tumors are produced by the tumor cells as well as by a variety of tumor-associated stromal cells, including fibroblasts, smooth muscle, and vascular cells, and also by cells of the immune system (Egeblad & Werb, 2002). Increased expression of MMPs is predictive of tumor aggressiveness, metastasis and low patient survival in lung, prostate, stomach, colon, breast, ovary, pancreatic, and oral squamous cell cancers (Yoshida et al. 2001; Kerkela & Saarialho-Kere, 2003; Mook et al. 2004; Jones et al. 2004; Katayama et al. 2004; Morgia et al. 2005; Illman et al. 2006; Somiari et al. 2006; Tetu et al. 2006; Liu et al. 2007; Wu et al. 2007). Irregular overexpression of MMPs has also been linked to metastasis of tumor cells in cancers including those of breast (Wolf et al. 1993; Freije et al. 1994), colon (Newell et al. 1994) and lung (Muller et al. 1991). It is noteworthy that there is not a single MMP that is consistently overexpressed in all tumor types, nor has a regular pattern of MMP expression been seen among the variety of human cancers (Kerkela & Saarialho-Kere, 2003). The expression of MMPs in cancer tumors mirror fundamental tissue heterogeneity, as various tumors express different subsets of ECM components, cell surface receptors, and cell tissue interactions. Nevertheless, is has been reported that MMP-1, -2, -3, -7, -9, -11 and MT1-MMP (MMP-14) are frequently overexpressed in many human tumors (Orlichenko & Radisky, 2008).

Cathepsins

Cathepsins are proteases which act on a wide range of ECM components, including proteoglycans and collagens. Cathepsins play a role in cancer invasion due to their ability to activate uPA (Kobayashi et al. 1991). During normal conditions cathepsins are controlled by their endogenous inhibitors; the cystatins superfamily of protease inhibitors (Calkins & Sloane, 1995). Modifications in the cathepsin versus inhibitor ratio are possibly involved in tumor progression and have been reported in numerous human cancers. Cathepsins degrade the ECM to facilitate growth and invasion into surrounding tissue and vasculature (Jedeszko & Sloane, 2004). In the literature, there is evidence of their functional role in tumor growth, migration, invasion, angiogenesis and metastasis (Mohamed & Sloane, 2006). It has been shown that the levels and localization of cathepsins and their inhibitors may be of diagnostic and prognostic value in many types of cancer (Jedeszko & Sloane, 2004; Mohamed & Sloane, 2006).

ECM proteins

The ECM does not only act as a barrier but it also serves as a passive and active substrate for migrating cells. It presents signaling functions itself and acts as a protein deposit. Thus the ECM is associated with a large number of proteins some of which are implicated in cancer progression and therapy response which may play an important role in cancer prognosis.

The ECM contains collagen types I and III, galectins, proteoglycans such as heparin sulphate and hyaluronic acid, and glycoproteins such as fibronectin, fibulins, and tenascin C. Collagen type IV, laminin, entactin, and certain proteoglycans are distinctively localized in the BM which divide organ parenchymal cells from the interstitial stroma (Figure 1; Van et al. 2000). In relation to tumor growth, the fibrillar collagens (types I, II, III, V and IX) normally have a low turnover but their metabolism is increased during the ECM remodeling that characterizes tumor evolution (Tlsty & Coussens, 2006).

Collagen type I is the most abundant protein of the interstitial ECM, which is composed of two α1 chains and one α2 chain that pack together into thin fibrils. Crosslinking of collagen with other proteins is mediated *via* lysyl oxidase (LOX). The glycoproteins fibronectin and tenascin C modulate the integrin-mediated adhesion of cells to other ECM proteins, for example, collagens, and play as such a key role in cancer invasion. A single gene encodes fibronectin but alternative splicing allows formation of multiple isoforms from which some are tumor specific (Kaspar et al. 2006). The fibulins, Galectin-1 and Fibulin-1 function as intramolecular bridges in the organization of ECM supramolecular structures, such as elastic fibers and basement membranes(Gallagher et al. 2005). Galectin-1 and Fibulin-1 can bind ECM components, that is, laminin, fibronectin, and therefore modify the adhesive properties of cancer cells (Gallagher et al. 2005; Rabinovich, 2005; Camby et al. 2006). The proteoglycan, heparan sulphate, is a core protein in the network of macromolecules representing the ECM. The side chains of heparan sulphate are cleaved off by heparanase, an endoglucuronidase, resulting in fragments of 5-7 kDa in size (Vlodavsky et al. 2006). As a result, the integrity of the ECM is affected, and proinflammatory, proangiogenic and prometastatic factors from the ECM are released (Vlodavsky et al. 2007).

Indeed, the synthesis, concentration and circulating levels (serum concentration) of degradation products of type I collagen have been proven to be increased during breast, bone, lung, ovarian, prostate and skin malignancy (Zhu et al. 1995; Santala et al. 1999; Ylisirnio et al. 1999; Ylisirnio et al. 2001; Jussila et al. 2004; Parikka et al. 2005).

Matrix composition affects cell phenotype

The importance of matrix stiffness in tissue-specific differentiation is exemplified by the fact that cells grown as monolayers (two dimensional: 2D) on top of either a plastic substrate or a glass cover slip, with or without ECM ligand, fail to assemble the same tissue-like structures as those growing in normal ECM (3D). Cells growing on plastic or glass are also unable to express differentiated proteins upon stimulation (Paszek et al.

2005), or respond to growth factors or protease inhibitors in the same way as cells growing in a three-dimensional setting (Karsdal et al. 2002). These phenotypic disparities can be explained, in part, by the fact that living tissues in 3D emit biological signals that may be read by specific integrins, but this signaling is nonexistent in 2D substrata such as tissue-culture plastic. Another illustration of this phenomenon is that when epithelial cells and melanocytes are grown in a 3D ECM microenvironment, they assemble into tissue-like structures and express differentiated proteins when given the correct soluble stimuli (Haass et al. 2004). Neither behavior is seen when the same cells are cultured on 2D plastic substrata.

The architecture of the interstitial tissue matrix *in vivo* also differs substantially from that found typically in tissues cultured on plastic, and this too can have dramatic effects on cell behavior (Karsdal et al. 2002). For instance, osteoblasts grown on plastic in 2D do not rely on MMPs for survival, whereas osteoblasts embedded in an interstitial matrix, such as 3D type I collagen, are critically dependent for their survival on MMP-activation of latent TGF-beta (Karsdal et al. 2002). Thus, the matrix architecture is crucial to the phenotype and survival of cells. Interestingly, the orientation of collagen fibers can critically regulate cell and tissue behavior (O'Brien et al. 2001; Pedersen & Swartz, 2005; Pedersen et al. 2010). This 3D contextual information is lost when cells are grown in 2D.

Varying components of the ECM also influence the ability of the matrix to regulate cell and tissue behavior. The ECM transmits signals through various specialized cell-membrane receptors including integrins, Discoid Domain Receptors (DDRs) and syndecans(Hynes, 2002, 2003, 2004, 2007, 2009). Integrins are an excellent model on how an altered ECM could promote tumor progression. Integrins consist of 24 distinct transmembrane heterodimers that relay cues from the surrounding ECM to regulate cell growth, survival, motility, invasion and differentiation (Hynes, 2002, 2003, 2004, 2007, 2009). They are able to interact with the ECM externally, with cytoplasmic adhesion plaque proteins and the cytoskeleton intracellularly to influence cell behavior. Integrin-ECM interactions regulate cell fate by activating multiple biochemical signaling circuits and altering cell shape (Clark & Brugge, 1995; Clark et al. 1998). This occurs either through direct interactions between ECM receptors and actin-linked proteins or cytoskeletal reorganization induced by activating cytoskeletal remodeling enzymes, such as RhoGTPases (Clark & Brugge, 1995; Clark et al. 1998).

This section highlights that the composition of the ECM affects the phenotype of cells though specific receptor-mediated interactions. Certain ECM compositions and structures results in contexts-dependent response to given stimuli, which is absent in other experimental settings.

PTMs in the ECM

PTMs are modifications to the composition or structure of proteins, which are noncoded, and unique parts of a molecule know as neoepitopes (Karsdal et al. 2010). Pathologically relevant protein modifications are not restricted to protease activity, although the subpopulation of neoepitopes generated through this mechanism may be of paramount importance. Figure 3 depicts a handful of different types of PTMs. Some have been identified and used as biochemical markers as a measure of the disease activity (Karsdal et al. 2009), and also as contributions to disease process(Karsdal et al. 2010), as they change the functionality of the proteins.

Today, it is well established that PTMs can uncover cryptic epitopes and/or create novel epitopes, that may initiate autoimmune reactions (Cloos & Christgau, 2004). Antigenicity and interactions of proteins with components of the immune system are possibly affected by PTMs, and modified self-antigens may be nontolerated during early T-cell selection and trigger reactions by the immune system. In turn, this may play a role in the initiation and pathogenesis of autoimmune diseases (Takahashi et al. 1997; Chen et al. 1998; Miyata et al. 1998; Cloos & Jensen, 2000; DeGroot et al. 2001; Ahsan et al. 2003; Saudek & Kay, 2003; Cloos & Christgau, 2004; Senolt et al. 2005; Yoshida et al. 2005; Kurien & Scofield, 2006; Sheikh et al. 2007; Griffiths, 2008; Choi & Lim, 2009; Kralev et al. 2009; Schwartz et al. 2009; Sjoberg & Bulterijs, 2009; Yamamoto et al. 2009). These PTMs may be both early markers as well as pathological events leading to cancer and chronic inflammation (Marx, 2004; Tlsty & Coussens, 2006; Riehl et al. 2009; Schetter et al. 2010). Regardless of whether PTMs are the chicken or the egg, the examples presented in this paper further emphasize that PTMs are relevant markers of cancer pathogenesis. Assays developed to detect neoepitopes may aid the understanding of the temporal events leading to PTMs and their role in disease mechanisms. In the following section, some of these PTM are described.

Cross-linking

Cross-linking, depicted in Figure 3A, plays an important role in the ECM meshwork and thereby in tissue integrity. Cross-linking between different ECM components or between different protein chains can result from enzymatic and nonenzymatic pathways. Enzymatic cross-

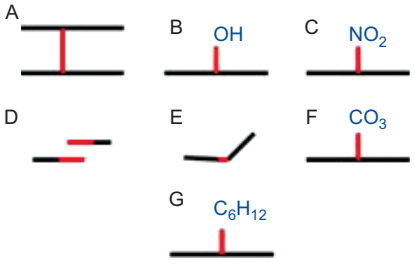

Figure 3. Different PTMs: (A) Cross-linking occurring between proteins/protein chains; (B) Hydroxylation of prolines (oxidation); (C) Nitrosylation of tyrosines (oxidation); (D) Protease-generated fragments creating free ends; (E) Isomerization of aspartate changing the peptide conformation; (F) Glycosylation generating sugar chains; (G) Citrullination of arginine.

linking is often processed by the enzyme LOX, which has been shown to promote the linearization of interstitial collagens, stiffing the tissues, and thus leads to neoplastic progression of tumor cells (Erler et al. 2006; Kass et al. 2007; Erler & Weaver, 2009; Levental et al. 2009). Interestingly, this matrix stiffness was associated with different phenotypes and enhanced mechanoresponsiveness of the epithelium (Erler et al. 2006; Erler & Weaver, 2009). This highlights that PTM plays an important part in both the initiation and progression of metastasis.

Within both the bone and cartilage, field assays have been developed using antibodies very specific for protease-cleaved sites in type I collagen (Rosenquist et al. 1998) and type II collagen (Oestergaard et al. 2006), respectively. These assays also assess the cross-linking between the lysines in the epitopes assesses by the antibody. These assays have proven valuable for the evaluation of bone- and cartilage-related diseases (Schaller et al. 2005). CTX-II for cartilage is thoroughly described in the section "Protease generated neoepitopes" in this paper. CTX-I is an 8-amino acid fragment from the C-telopeptide of type I collagen generated by cathepsin K activity and the rate of its release from bone is a useful reflection of the resorbing activity of osteoclasts (Bone, 1992) thus being useful for the evaluation of treatment efficacy in, for example, osteoporosis (OP; Leeming et al. 2006a). The CTX epitope contains an aspartyl-glycine motif (DG) that is prone to spontaneous isomerization. In other words, EKAHD(α)GGR epitopes are released during degradation of newly synthesized type I collagen, whereas EKAHD(β)GGR epitopes are released from matured collagen type I. It has been established that the α/β ratio is a useful measure of the age of bone tissue (Byrjalsen et al. 2008; Karsdal et al. 2008a; Leeming et al. 2009); the lower is the ratio, the older is the bone tissue (Fledelius et al. 1997). Resorption rate of newly synthesized collagen type I can be assessed by specific immunoassays targeting the detection of αCTX in urine samples (Cloos et al. 2004). Degradation rate of matured, isomerized collagen can be estimated by another specific assay targeting βCTX in both urine and serum samples (Rosenquist et al. 1998).

Oxidations and hydroxylations

Oxidative damage to proteins is often caused by the action of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydrogen peroxide and nitric oxide (NO) generated in cells by the mitochondrial respiratory chain (Poyton et al. 2009). Oxidizing PTMs have been implicated in several pathological and healthy tissue turnover processes (Figures 1B and 1C). Although many amino acids can be attacked by ROS, some seem more likely to undergo oxidation than others. For example, lysine and proline are readily oxidized to aldehydes; sulfoxidation of methionine; and nitrosylation of tyrosines (Cloos & Christgau, 2002). Under normal conditions these ROSs are strictly regulated by antioxidants, such as peroxidases and dimutases among others (Kowluru et al. 2006). However, under pathological conditions oxidation may be implicated in tissue destruction. The role of ROS, in almost all aspects of cancer initiation and development (Colell et al. 2009; DeNicola & Tuveson, 2009; Faux et al. 2009; Poyton et al. 2009; Weinberg & Chandel, 2009; Chaiswing & Oberley, 2010; Lu et al. 2010; Schetter et al. 2010) is still debated. Measurement of specific components of the ECM that hold these PTMs may be used for both early diagnostic and prognosis of cancer.

Protease-generated neoepitopes

Matrix remodeling at specific disease stages results in both elevated levels of, and uniquely modified, proteins. Endopeptidases, such as MMPs and cysteine proteases, play major roles in the degradation of extracellular macromolecules such as collagens and proteoglycans (Figure 3D). Specific proteolytic activities are a prerequisite for a range of cellular functions and interactions with the ECM resulting in the generation of specific cleavage fragments. Even though many components of the ECM, as well as enzymes responsible for remodeling, are present in different tissues, the combination of a specific peptidase and specific ECM proteins may provide a unique combination that elucidates activity in a particular tissue or a specific disease mechanism.

One often taught example of protease degradation of a given tissue is that of joint degenerative diseases. Joint degenerative diseases lead to alterations in the metabolism of the articular cartilage and subchondral bone (Bailey & Mansell, 1997; Dieppe, 1999; Felson & Neogi, 2004; Anderson-MacKenzie et al. 2005; Karsdal et al. 2008b, 2008c). Cartilage is for the most part composed of collagen type II, which accounts for 60% to 70% of the dry weight of cartilage, and proteoglycans accounting for 10% of the dry weight, of which aggrecan is the most abundant (Sondergaard et al. 2006). As 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. 2005; Karsdal et al. 2009). CTX-II is an MMP-generated neoepitope derived from the C-terminal part of type II collagen (Sondergaard et al. 2006), and measurement of CTX-II is highly useful for monitoring degradation of type II collagen in experimental set-ups assessing cartilage degradation (Karsdal et al. 2007; Karsdal et al. 2008c). Examples of 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 types I and II as well as aggrecan are the most described. Assays detecting a few neoepitopes that have been developed and are used in both clinical and preclinical studies were reviewed recently (Qvist et al. 2010).

As many cancers are present in soft tissues of intestines and BM, identification of neoepitopes from abundant

proteins from those tissues may be a reasonable approach. To some extent this has been done for ICTP, and MMP-derived fragment of type I collagen (Ylisirnio et al. 1999; Simojoki et al. 2001; Ylisirnio et al. 2001; Garnero et al. 2003; Santala et al. 2004). In alignment, a range of biochemical markers based on degradation products of the ECM may be identified and used in cancer, particularly, collagen is the interstitial or basement membranes that are the host tissue for many cancer types. In particular, the collagen composition of the basement membrane and interstitial matrix, may be relevant for the development of given marker for the ECM remodeling associated with soft tissue metastasis. This concept is illustrated in Figure 4.

Isomerization: age of ECM proteins

Proteins containing aspartate (D), asparagine (N), glutamate (E), or glutamine (Q) residue linked to a low-molecular weight amino acid, such as glycine (G), can undergo spontaneous nonenzymatic isomerization

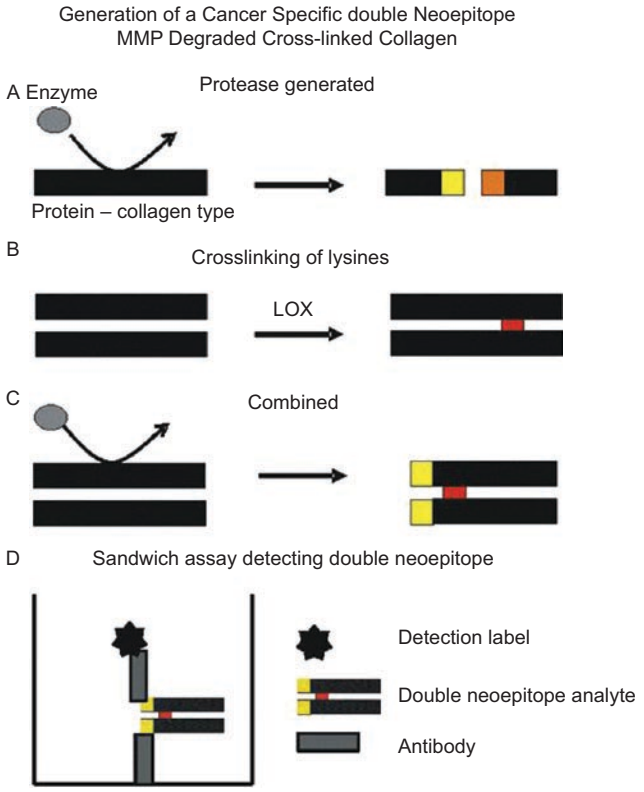

Figure 4. (A) An enzyme, most likely an MMP, cleaves a collagen molecules. This generates a cut in the peptide sequence, exposing an N and C terminal truncated molecule. (B) One of the most unregulated enzymes in cancer is the lysyl oxidase (LOX). This enzyme crosslinks the lysines of the collagen chain resulting in a more stiffened tissue. In the local area of cancers metastasis and growth, these processes are occurring at a more rapid pace, then other parts in the body, with increased expression of a range of proteases, collagen and other enzymes. (C) When an enzyme cleaves collagen that has been crosslinked by LOX a double neoepitope is generated. (D) The double neoepitope can be detected in a sandwich assay by the use of the same antibody as the catcher antibody and the detection antibody.

(Cloos & Christgau, 2004). This isomerization introduces a kink in the conformation of the molecule, as the peptide backbone is redirected from the α-carboxyl group in the native newly synthesized form to the side chain β-carboxyl (Fledelius et al. 1997; Figure 3E). Peptides that contain amino acid isomerizations are often resistant to proteolysis (Johnson & Aswad, 1990; Cloos & Fledelius, 2000) and this feature affects the procession of antigens for presentation on the major histocompatibility complex II (MHC-II) involved in the immune-response signaling for the production of T-cells and antibodies (Cloos & Christgau, 2004). In preclinical studies it has been shown that various known auto-antigens contain sites prone to deamidation and isomerization involved in type I diabetes, rheumatoid arthritis, systemic lupus erythematosus and experimental autoimmune encephalomyelitis (Voorter et al. 1988; Brange et al. 1992; Mamula et al. 1999; Cloos & Fledelius, 2000; Young et al. 2001). The C-telopeptide collagen type I marker CTX-I is a marker of bone resorption. It has been shown that assessment of the nonisomerized epitope (ALPHA CTX-I) is more sensitive as a marker for bone metastases secondary to breast and prostate cancer as compared to the isomerized epitope (BETA CTX-I; Leeming et al. 2006c). This is due to the high ECMR of collagen type I in the bone area invaded by cancer cells and thus a high amount of newly formed nonisomerized collagen type I is undergoing resorption by osteoclasts in this high-turnover situation.

Nonenzymatic glycosylation

Nonenzymatic glycosylation is also called the Maillard reaction, and leads to PTM of proteins, nucleic acids and lipids (Lapolla et al. 2005; Figure 1F). A common cause of nonenzymatic glycosylation is increased blood glucose levels, and accordingly most knowledge about nonenzymatic glycosylation arises from studies performed in diabetics(Lapolla et al. 2005). The marker HbA1c is an established PTM marker in type II diabetes. Recently, advanced glycation end products (AGEs) have been implicated in cancers. The chemical-induced—that is nicotine—accumulation of AGEs is an inducer of cancer (Treweek et al. 2009). Furthermore, the receptor for AGEs, called RAGE, is currently under intense investigation as both a marker and an inducer of cancer (Riehl et al. 2009), linking chronic inflammation and cancer (Johansen, 2006; Tlsty & Coussens, 2006; Riehl et al. 2009; Schetter et al. 2010).

Citrullination

Citrullination or deimination is the term used for the PTM of the amino acid arginine which can transform into the amino acid citrulline (Figure 1G). The change is facilitated by peptidylarginine deiminases (PADs; Gyorgy et al. 2006; Anzilotti et al. 2010). The conversion of arginine into citrulline can have important consequences for the structure and function of proteins, as arginine is positively charged at a neutral pH, whereas citrulline

is uncharged. This increases the hydrophobicity of the protein, leading to changes in protein folding.

Histone deacetylase 1 (HDAC1) inhibitors are currently under development for certain cancer diseases, in particular breast cancer (Krueger & Srivastava, 2006). Histone lysine and arginine residues are subject to a wide array of PTMs including methylation, citrullination, acetylation, ubiquitination, and sumoylation. The combined action of these modifications regulates critical DNA processes including replication, repair, and transcription. In addition, enzymes that modify histone lysine and arginine residues have been correlated with a variety of human diseases including arthritis, cancer, heart disease, diabetes, and neurodegenerative disorders (Chang & Han, 2006; Chang et al. 2009).

Histone methylation plays key roles in regulating chromatin structure and function. The recent identification of enzymes that antagonize or remove histone methylation offers new insights into histone methylation plasticity in the regulation of epigenetic pathways. Peptidylarginine deiminase 4 (PADI4; also known as PAD4) was the first enzyme shown to antagonize histone methylation. PADI4 functions as a histone deiminase converting a methylarginine residue to citrulline at specific sites on the tails of histones H3 and H4. PADI4 associates with the HDAC1 (Chang & Han, 2006; Chang et al. 2009; Smith & Denu, 2009).

This highlights this class of PTMs, whether cellular or noncellular, are key signaling points in the initiation and pathogenesis of cancer. Importantly, the same protein modification may both serve as a target for drug development and as a biochemical marker target.

An example of a combined aged, cross-linked and cleaved neoepitope for the evaluation of bone metastases

The relationship between skeletal tumor load and elevations in serum or urine levels of ALPHA CTX and seven other biomarkers related to bone turnover have been investigated in a pooled group of breast and prostate cancer patients (Leeming et al. 2006c). Patients were stratified according to the Soloway score: Score 0 = 0 bone metastases; Score 1 = <6 bone metastases; Score 2 = 6-20 bone metastases; Score 3 = >20 bone metastases; Score 4 = superscan where >75 % ribs, vertebrae and pelvic bone are infected. In breast cancer patients, a strong linear association was observed between bone metastases and all biomarkers except osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) (Figure 5). All six remaining markers were significantly elevated in patients with Soloway Score 1. The relative percentage increases in biomarker levels in the presence of bone metastases was most pronounced for ALPHA CTX-I, which was elevated by more than 600% at Soloway Score 3. The next highest increases were in bone-specific alkaline phosphatase (BSAP) and N-telopeptide of collagen type I (NTX) which were elevated by 470% and 440% at Soloway Score 3, respectively. This finding was supported by observations in prostate cancer patients which showed that of seven biomarkers, ALPHA CTX-I was the most sensitive for bone metastases (Garnero et al. 2000). The higher sensitivity of ALPHA CTX-I could be explained by the fact that this epitope is released from sites of high bone remodeling, where collagen fibrils do not have time to mature and undergo β-isomerization. Furthermore, the ALPHA CTX epitope was located by immunostaining in adjacent sections of bones invaded by breast cancer or prostate cancer (Leeming et al. 2006b), and at the sites of high bone remodeling.

Finally, ALPHA CTX has been proven to be more useful for the evaluation of bone metastases in a longitudinal study of prostate cancer patients than prostate-specific antigen (PSA) and total alkaline phosphatase (tALP; Leeming et al. 2008). PSA was elevated in both lymph node-negative and -positive patients compared to healthy age-matched controls, while ALPHA CTX was elevated only in lymph node-positive patients. tALP levels were similar across the groups. In a second arm of this study, patients were treated with docetaxel alone or docetaxel and zoledronic acid combined. PSA and tALP levels decreased from baseline values in patients with and without bone metastases who received either treatment regimen, indicating that docetaxel or docetaxel/zoledronate treatment had similar effects on these markers. In contrast, ALPHA CTX did not decrease with docetaxel treatment in the negative bone-metastases group compared to baseline while it decreased significantly with docetaxel/

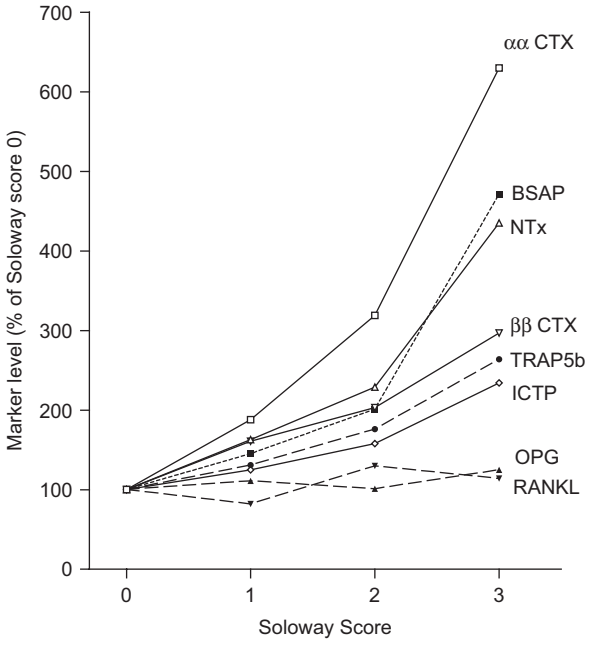

Figure 5. Relative increases in bone resorption, bone formation and osteoclastogenesis marker levels as a function of the extent of skeletal involvement, assessed in 132 patients with breast or prostate cancer. Relative increases are expressed as a percentage of levels in patients with a Soloway Score 0 (Leeming et al. 2006c).

zoledronate treatment in the positive bone-metastases group. This suggests that ALPHA CTX is superior to PSA and tALP for identifying patients at high risk of metastatic disease and for monitoring progression of bone metastases in prostate cancer patients during treatment.

These data support, that careful selection of matrix constituents and in particular those that carry one or more PTMs such as isomerization in a collagen type I fragment generated by cathepsin K as described for this example, may be superior markers to reflect pathological, including malignant, events in the ECM.

Future direction

In this manuscript, we have highlighted developments in protein chemistry, namely the combination of multiple disease-specific neoepitopes that could be applied to clinical chemistry. The combination of multiple neoepitopes as biomarkers as already been applied to some diseases, and this approach may be advantageous in other disease areas such as cancer which also involves highly remodeled tissues. By incorporating the most optimal biochemical markers in all aspects of drug discovery and development, novel treatment opportunities may be identified, and their clinical development streamlined by the ease of early detection of both efficacy and safety concerns.

As illustrated in Figure 5, cancer cells invade the matrix by expressing a battery of proteolytic enzymes. These enzymes degrade the ECM and a range of other PTMs as described, releasing smaller fragment of proteins of the ECM into the circulation. An optimal biochemical marker may be designed by identifying the common denominator of specific pathophysiological processes to determine the marker tissue specificity and sensitivity. Different cancer cells predominately express given proteases that in combination with different signature proteins from different host tissues, may provide optimal selectivity of that tissue-cancer cell combination. By carefully examining these relationships, a biomarker may be identified. Biochemical markers based on the advanced disease/tissue neoepitope approach could become an important tool to be used in combination with others for diagnosing and staging disease as well as assessing efficacy and safety of new therapeutic interventions.

Declaration of interest

Leeming DJ, Bay-Jensen AC, Vassiliadis E, Henriksen K and Karsdal MA are full time employees at Nordic Bioscience. The authors acknowledge the funding from the Danish "Ministry of Science, Technology and Science" and the Danish Science Foundation (*Den Danske Forskningsfond*).

References

Ahsan H, Ali A, Ali R. (2003). Oxygen free radicals and systemic autoimmunity. *Clin Exp Immunol* 131:398–404.

Anderson-MacKenzie JM, Quasnichka HL, Starr RL, Lewis EJ, Billingham ME, Bailey AJ. (2005). Fundamental subchondral bone changes in spontaneous knee osteoarthritis. *Int J Biochem Cell Biol* 37:224–236.

Anzilotti C, Pratesi F, Tommasi C, Migliorini P. (2010). Peptidylarginine deiminase 4 and citrullination in health and disease. *Autoimmun Rev* 9:158–160.

Aumailley M, Gayraud B. (1998). Structure and biological activity of the extracellular matrix. *J Mol Med* 76:253–265.

Bailey AJ, Mansell JP. (1997). Do subchondral bone changes exacerbate or precede articular cartilage destruction in osteoarthritis of the elderly? *Gerontology* 43:296–304.

Bissell MJ, Aggeler J. (1987). Dynamic reciprocity: how do extracellular matrix and hormones direct gene expression? *Prog Clin Biol Res* 249:251–262.

Bissell MJ, Radisky D. (2001). Putting tumours in context. *Nat Rev Cancer* 1:46–54.

Blood CH, Zetter BR. (1990). Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochim Biophys Acta* 1032:89–118.

Bone HG. (1992). The future of osteoporosis diagnosis and therapy. *Ann Ital Med Int* 7:166S–170S.

Bosman FT, Stamenkovic I. (2003). Functional structure and composition of the extracellular matrix. *J Pathol* 200:423–428.

Bosques CJ, Raguram S, Sasisekharan R. (2006). The sweet side of biomarker discovery. *Nat Biotechnol* 24:1100–1101.

Brange J, Langkjaer L, Havelund S, Volund A. (1992). Chemical stability of insulin. 1. Hydrolytic degradation during storage of pharmaceutical preparations. *Pharm Res* 9:715–726.

Byrjalsen I, Leeming DJ, Qvist P, Christiansen C, Karsdal MA. (2008). Bone turnover and bone collagen maturation in osteoporosis: effects of antiresorptive therapies. *Osteoporos Int* 19:339–348.

Calkins CC, Sloane BF. (1995). Mammalian cysteine protease inhibitors: biochemical properties and possible roles in tumor progression. *Biol Chem Hoppe-Seyler* 376:71–80.

Camby I, Le Mercier M, Lefranc F, Kiss R. (2006). Galectin-1: a small protein with major functions. *Glycobiology* 16:137R–157R.

Chaiswing L, Oberley TD. (2010). Extracellular/microenvironmental redox state. *Antioxid Redox Signal* 13:449–465.

Chang X, Han J. (2006). Expression of peptidylarginine deiminase type 4 (PAD4) in various tumors. *Mol Carcinog* 45:183–196.

Chang X, Han J, Pang L, Zhao Y, Yang Y, Shen Z. (2009). Increased PADI4 expression in blood and tissues of patients with malignant tumors. *BMC Cancer* 9:40.

Chen JR, Takahashi M, Suzuki M, Kushida K, Miyamoto S, Inoue T. (1998). Pentosidine in synovial fluid in osteoarthritis and rheumatoid arthritis: relationship with disease activity in rheumatoid arthritis. *J Rheumatol* 25:2440–2444.

Choi YG, Lim S. (2009). Characterization of anti-advanced glycation end product antibodies to nonenzymatically lysine-derived and arginine-derived glycated products. *J Immunoassay Immunochem* 30:386–399.

Clarijs R, Ruiter DJ, De Waal RM. (2003). Pathophysiological implications of stroma pattern formation in uveal melanoma. *J Cell Physiol* 194:267–271.

Clark EA, Brugge JS. (1995). Integrins and signal transduction pathways: the road taken. *Science* 268:233–239.

Clark EA, King WG, Brugge JS, Symons M, Hynes RO. (1998). Integrin-mediated signals regulated by members of the rho family of GTPases. *J Cell Biol* 142:573–586.

Cloos PA, Christgau S. (2002). Non-enzymatic covalent modifications of proteins: mechanisms, physiological consequences and clinical applications. *Matrix Biol* 21:39–52.

Cloos PA, Christgau S. (2004). Post-translational modifications of proteins: implications for aging, antigen recognition, and autoimmunity. *Biogerontology* 5:139–158.

Cloos PA, Fledelius C. (2000). Collagen fragments in urine derived from bone resorption are highly racemized and isomerized: a biological clock of protein aging with clinical potential. *Biochem J* 345 Pt 3:473–480.

Cloos PA, Jensen AL. (2000). Age-related de-phosphorylation of proteins in dentin: a biological tool for assessment of protein age. *Biogerontology* 1:341–356.

Cloos PA, Lyubimova N, Solberg H, Qvist P, Christiansen C, Byrjalsen I, Christgau S. (2004). An immunoassay for measuring fragments of newly synthesized collagen type I produced during metastatic invasion of bone. *Clin Lab* 50:279–289.

Colell A, Green DR, Ricci JE. (2009). Novel roles for GAPDH in cell death and carcinogenesis. *Cell Death Differ* 16:1573–1581.

Condeelis J, Pollard JW. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124:263–266.

DeGroot J, Verzijl N, Wenting-Van Wijk MJ, Bank RA, Lafeber FP, Bijlsma JW, TeKoppele JM. (2001). Age-related decrease in susceptibility of human articular cartilage to matrix metalloproteinase-mediated degradation: the role of advanced glycation end products. *Arthritis Rheum* 44:2562–2571.

DeNicola GM, Tuveson DA. (2009). RAS in cellular transformation and senescence. *Eur J Cancer* 45 Suppl 1:211–216.

Dieppe P. (1999). Subchondral bone should be the main target for the treatment of pain and disease progression in osteoarthritis. *Osteoarthr Cartil* 7:325–326.

Dolberg DS, Bissell MJ. (1984). Inability of Rous sarcoma virus to cause sarcomas in the avian embryo. *Nature* 309:552–556.

Duffy MJ. (1996). The biochemistry of metastasis. *Adv Clin Chem* 32:135–166.

Egeblad M, Werb Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2:161–174.

Erler JT, Bennewith KL, Nicolau M, Dornhöfer N, Kong C, Le QT, Chi JT, Jeffrey SS, Giaccia AJ. (2006). Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* 440:1222–1226.

Erler JT, Weaver VM. (2009). Three-dimensional context regulation of metastasis. *Clin Exp Metastasis* 26:35–49.

Faux SP, Tai T, Thorne D, Xu Y, Breheny D, Gaca M. (2009). The role of oxidative stress in the biological responses of lung epithelial cells to cigarette smoke. *Biomarkers* 14 Suppl 1:90–96.

Felson DT, Neogi T. (2004). Osteoarthritis: is it a disease of cartilage or of bone? *Arthritis Rheum* 50:341–344.

Fledelius C, Johnsen AH, Cloos PA, Bonde M, Qvist P. (1997). Characterization of urinary degradation products derived from type I collagen. Identification of a beta-isomerized Asp-Gly sequence within the C-terminal telopeptide (alpha1) region. *J Biol Chem* 272:9755–9763.

Freije JM, Díez-Itza I, Balbín M, Sánchez LM, Blasco R, Tolivia J, López-Otín C. (1994). Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J Biol Chem* 269:16766–16773.

Gallagher WM, Currid CA, Whelan LC. (2005). Fibulins and cancer: friend or foe? *Trends Mol Med* 11:336–340.

Garnero P, Buchs N, Zekri J, Rizzoli R, Coleman RE, Delmas PD. (2000). Markers of bone turnover for the management of patients with bone metastases from prostate cancer. *Br J Cancer* 82:858–864.

Garnero P, Ferreras M, Karsdal MA, NicAmhlaoibh R, Risteli J, Borel O, Qvist P, Delmas PD, Foged NT, Delaisse JM. (2003). The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. *J Bone Miner Res* 18:859–867.

Griffiths HR. (2008). Is the generation of neo-antigenic determinants by free radicals central to the development of autoimmune rheumatoid disease? *Autoimmun Rev* 7:544–549.

György B, Tóth E, Tarcsa E, Falus A, Buzás EI. (2006). Citrullination: a posttranslational modification in health and disease. *Int J Biochem Cell Biol* 38:1662–1677.

Haass NK, Smalley KS, Herlyn M. (2004). The role of altered cell-cell communication in melanoma progression. *J Mol Histol* 35:309–318.

Hanash SM, Pitteri SJ, Faca VM. (2008). Mining the plasma proteome for cancer biomarkers. *Nature* 452:571–579.

Hynes RO. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* 110:673–687.

Hynes RO. (2003). Structural biology. Changing partners. *Science* 300:755–756.

Hynes RO. (2004). The emergence of integrins: a personal and historical perspective. *Matrix Biol* 23:333–340.

Hynes RO. (2007). Cell-matrix adhesion in vascular development. *J Thromb Haemost* 5 Suppl 1:32–40.

Hynes RO. (2009). The extracellular matrix: not just pretty fibrils. *Science* 326:1216–1219.

Illman SA, Lehti K, Keski-Oja J, Lohi J. (2006). Epilysin (MMP-28) induces TGF-beta mediated epithelial to mesenchymal transition in lung carcinoma cells. *J Cell Sci* 119:3856–3865.

Ingber DE. (2008). Can cancer be reversed by engineering the tumor microenvironment? *Semin Cancer Biol* 18:356–364.

Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW. (2006). Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev Dyn* 235:3222–3229.

Jedeszko C, Sloane BF. (2004). Cysteine cathepsins in human cancer. *Biol Chem* 385:1017–1027.

Johansen JS. (2006). Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. *Dan Med Bull* 53:172–209.

Johnson BA, Aswad DW. (1990). Fragmentation of isoaspartyl peptides and proteins by carboxypeptidase Y: release of isoaspartyl dipeptides as a result of internal and external cleavage. *Biochemistry* 29:4373–4380.

Jones LE, Humphreys MJ, Campbell F, Neoptolemos JP, Boyd MT. (2004). Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival. *Clin Cancer Res* 10:2832–2845.

Jussila T, Kauppila S, Bode M, Tapanainen J, Risteli J, Risteli L, Kauppila A, Stenbäck F. (2004). Synthesis and maturation of type I and type III collagens in endometrial adenocarcinoma. *Eur J Obstet Gynecol Reprod Biol* 115:66–74.

Karsdal MA, Byrjalsen I, Leeming DJ, Delmas PD, Christiansen C. (2008a). The effects of oral calcitonin on bone collagen maturation: implications for bone turnover and quality. *Osteoporos Int* 19:1355–1361.

Karsdal MA, Henriksen K, Leeming DJ, Mitchell P, Duffin K, Barascuk N, Klickstein L, Aggarwal P, Nemirovskiy O, Byrjalsen I, Qvist P, Bay-Jensen AC, Dam EB, Madsen SH, Christiansen C. (2009). 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. *Biomarkers* 14:181–202.

Karsdal MA, Henriksen K, Leeming DJ, Woodworth T, Vassiliadis E, Bay-Jensen AC. (2010). Novel combinations of Post-Translational Modification (PTM) neo-epitopes provide tissue-specific biochemical markers–are they the cause or the consequence of the disease? *Clin Biochem* 43:793–804.

Karsdal MA, Larsen L, Engsig MT, Lou H, Ferreras M, Lochter A, Delaissé JM, Foged NT. (2002). Matrix metalloproteinase-dependent activation of latent transforming growth factor-beta controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis. *J Biol Chem* 277:44061–44067.

Karsdal MA, Leeming DJ, Dam EB, Henriksen K, Alexandersen P, Pastoureau P, Altman RD, Christiansen C. (2008b). Should subchondral bone turnover be targeted when treating osteoarthritis? *Osteoarthr Cartil* 16:638–646.

Karsdal MA, Madsen SH, Christiansen C, Henriksen K, Fosang AJ, Sondergaard BC. (2008c). Cartilage degradation is fully reversible in the presence of aggrecanase but not matrix metalloproteinase activity. *Arthritis Res Ther* 10:R63.

Karsdal MA, Sumer EU, Wulf H, Madsen SH, Christiansen C, Fosang AJ, Sondergaard BC. (2007). Induction of increased cAMP levels in articular chondrocytes blocks matrix metalloproteinase-mediated cartilage degradation, but not aggrecanase-mediated cartilage degradation. *Arthritis Rheum* 56:1549–1558.

Kaspar M, Zardi L, Neri D. (2006). Fibronectin as target for tumor therapy. *Int J Cancer* 118:1331–1339.

Kass L, Erler JT, Dembo M, Weaver VM. (2007). Mammary epithelial cell: influence of extracellular matrix composition and organization during development and tumorigenesis. *Int J Biochem Cell Biol* 39:1987–1994.

Katayama A, Bandoh N, Kishibe K, Takahara M, Ogino T, Nonaka S, Harabuchi Y. (2004). Expressions of matrix metalloproteinases in early-stage oral squamous cell carcinoma as predictive indicators for tumor metastases and prognosis. *Clin Cancer Res* 10:634–640.

Kerkelä E, Saarialho-Kere U. (2003). Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. *Exp Dermatol* 12:109–125.

Kobayashi H, Schmitt M, Goretzki L, Chucholowski N, Calvete J, Kramer M, Günzler WA, Jänicke F, Graeff H. (1991). Cathepsin B efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (Pro-uPA). *J Biol Chem* 266:5147–5152.

Kowluru RA, Atasi L, Ho YS. (2006). Role of mitochondrial superoxide dismutase in the development of diabetic retinopathy. *Invest Ophthalmol Vis Sci* 47:1594–1599.

Kozaci LD, Buttle DJ, Hollander AP. (1997). Degradation of type II collagen, but not proteoglycan, correlates with matrix metalloproteinase activity in cartilage explant cultures. *Arthritis Rheum* 40:164–174.

Kralev S, Zimmerer E, Brueckmann M, Lang S, Kälsch T, Rippert A, Lin J, Borggrefe M, Hammes HP, Süselbeck T. (2009). Elevation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in patients presenting with acute myocardial infarction. *Clin Chem Lab Med* 47:446–451.

Krueger KE, Srivastava S. (2006). Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics. *Mol Cell Proteomics* 5:1799–1810.

Kurien BT, Scofield RH. (2006). Lipid peroxidation in systemic lupus erythematosus. *Indian J Exp Biol* 44:349–356.

Lapolla A, Traldi P, Fedele D. (2005). Importance of measuring products of non-enzymatic glycation of proteins. *Clin Biochem* 38:103–115.

Leeming DJ, Alexandersen P, Karsdal MA, Qvist P, Schaller S, Tankó LB. (2006a). An update on biomarkers of bone turnover and their utility in biomedical research and clinical practice. *Eur J Clin Pharmacol* 62:781–792.

Leeming DJ, Delling G, Koizumi M, Henriksen K, Karsdal MA, Li B, Qvist P, Tankó LB, Byrjalsen I. (2006b). Alpha CTX as a biomarker of skeletal invasion of breast cancer: immunolocalization and the load dependency of urinary excretion. *Cancer Epidemiol Biomarkers Prev* 15:1392–1395.

Leeming DJ, Hegele A, Byrjalsen I, Hofmann R, Qvist P, Karsdal MA, Schrader AJ, Wagner R, Olbert P. (2008). Biochemical markers for monitoring response to therapy: evidence for higher bone specificity by a novel marker compared with routine markers. *Cancer Epidemiol Biomarkers Prev* 17:1269–1276.

Leeming DJ, Henriksen K, Byrjalsen I, Qvist P, Madsen SH, Garnero P, Karsdal MA. (2009). Is bone quality associated with collagen age? *Osteoporos Int* 20:1461–1470.

Leeming DJ, Koizumi M, Byrjalsen I, Li B, Qvist P, Tankó LB. (2006c). The relative use of eight collagenous and noncollagenous markers for diagnosis of skeletal metastases in breast, prostate, or lung cancer patients. *Cancer Epidemiol Biomarkers Prev* 15:32–38.

Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL, Weaver VM. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* 139:891–906.

Li Z, Yasuda Y, Li W, Bogyo M, Katz N, Gordon RE, Fields GB, Bromme D. (2004). Regulation of collagenase activities of human cathepsins by glycosaminoglycans. *J Biol Chem* 279:5470–5479.

Liu D, Nakano J, Ishikawa S, Yokomise H, Ueno M, Kadota K, Urushihara M, Huang CL. (2007). Overexpression of matrix metalloproteinase-7 (MMP-7) correlates with tumor proliferation, and a poor prognosis in non-small cell lung cancer. *Lung Cancer* 58:384–391.

Lochter A, Bissell MJ. (1999). An odyssey from breast to bone: multi-step control of mammary metastases and osteolysis by matrix metalloproteinases. *Apmis* 107:128–136.

Lochter A, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ. (1997). Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol* 139:1861–1872.

Lü JM, Lin PH, Yao Q, Chen C. (2010). Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *J Cell Mol Med* 14:840–860.

Mamula MJ, Gee RJ, Elliott JI, Sette A, Southwood S, Jones PJ, Blier PR. (1999). Isoaspartyl post-translational modification triggers autoimmune responses to self-proteins. *J Biol Chem* 274:22321–22327.

Marx J (2004). Cancer research. Inflammation and cancer: the link grows stronger. *Science* 306:966–968.

Mintz B, Illmensee K. (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc Natl Acad Sci USA* 72:3585–3589.

Miyata T, Ishiguro N, Yasuda Y, Ito T, Nangaku M, Iwata H, Kurokawa K. (1998). Increased pentosidine, an advanced glycation end product, in plasma and synovial fluid from patients with rheumatoid arthritis and its relation with inflammatory markers. *Biochem Biophys Res Commun* 244:45–49.

Mohamed MM, Sloane BF. (2006). Cysteine cathepsins: multifunctional enzymes in cancer. *Nat Rev Cancer* 6:764–775.

Mook OR, Frederiks WM, Van Noorden CJ. (2004). The role of gelatinases in colorectal cancer progression and metastasis. *Biochim Biophys Acta* 1705:69–89.

Morgia G, Falsaperla M, Malaponte G, Madonia M, Indelicato M, Travali S, Mazzarino MC. (2005). Matrix metalloproteinases as diagnostic (MMP-13) and prognostic (MMP-2, MMP-9) markers of prostate cancer. *Urol Res* 33:44–50.

Muller D, Breathnach R, Engelmann A, Millon R, Bronner G, Flesch H, Dumont P, Eber M, Abecassis J. (1991). Expression of collagenase-related metalloproteinase genes in human lung or head and neck tumours. *Int J Cancer* 48:550–556.

Newell KJ, Witty JP, Rodgers WH, Matrisian LM. (1994). Expression and localization of matrix-degrading metalloproteinases during colorectal tumorigenesis. *Mol Carcinog* 10:199–206.

O'Brien LE, Jou TS, Pollack AL, Zhang Q, Hansen SH, Yurchenco P, Mostov KE. (2001). Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat Cell Biol* 3:831–838.

Oestergaard S, Chouinard L, Doyle N, Karsdal MA, Smith SY, Qvist P, Tankó LB. (2006). The utility of measuring C-terminal telopeptides of collagen type II (CTX-II) in serum and synovial fluid samples for estimation of articular cartilage status in experimental models of destructive joint diseases. *Osteoarthr Cartil* 14:670–679.

Orlichenko LS, Radisky DC. (2008). Matrix metalloproteinases stimulate epithelial-mesenchymal transition during tumor development. *Clin Exp Metastasis* 25:593–600.

Overall CM, López-Otín C. (2002). Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat Rev Cancer* 2:657–672.

Parikka V, Väänänen A, Risteli J, Salo T, Sorsa T, Väänänen HK, Lehenkari P. (2005). Human mesenchymal stem cell derived osteoblasts degrade organic bone matrix *in vitro* by matrix metalloproteinases. *Matrix Biol* 24:438–447.

Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell* 8:241–254.

Pedersen JA, Lichter S, Swartz MA. (2010). Cells in 3D matrices under interstitial flow: effects of extracellular matrix alignment on cell shear stress and drag forces. *J Biomech* 43:900–905.

Pedersen JA, Swartz MA. (2005). Mechanobiology in the third dimension. *Ann Biomed Eng* 33:1469–1490.

Poyton RO, Ball KA, Castello PR. (2009). Mitochondrial generation of free radicals and hypoxic signaling. *Trends Endocrinol Metab* 20:332–340.

Qvist P, Christiansen C, Karsdal MA, Madsen SH, Sondergaard BC, Bay-Jensen AC. (2010). Application of biochemical markers in development of drugs for treatment of osteoarthritis. *Biomarkers* 15:1–19.

Rabinovich GA. (2005). Galectin-1 as a potential cancer target. *Br J Cancer* 92:1188–1192.

Radisky DC, Bissell MJ. (2004). Cancer. Respect thy neighbor! *Science* 303:775–777.

Riehl A, Németh J, Angel P, Hess J. (2009). The receptor RAGE: Bridging inflammation and cancer. *Cell Commun Signal* 7:12.

Rosenquist C, Fledelius C, Christgau S, Pedersen BJ, Bonde M, Qvist P, Christiansen C. (1998). Serum CrossLaps One Step ELISA. First application of monoclonal antibodies for measurement in serum of bone-related degradation products from C-terminal telopeptides of type I collagen. *Clin Chem* 44:2281–2289.

Roycik MD, Fang X, Sang QX. (2009). A fresh prospect of extracellular matrix hydrolytic enzymes and their substrates. *Curr Pharm Des* 15:1295–1308.

Ruiter D, Bogenrieder T, Elder D, Herlyn M. (2002). Melanoma-stroma interactions: structural and functional aspects. *Lancet Oncol* 3:35–43.

Santala M, Risteli J, Kauppila A. (2004). Comparison of carboxyterminal telopeptide of type I collagen (ICTP) and CA 125 as predictors of prognosis in ovarian cancer. *Anticancer Res* 24:1057–1062.

Santala M, Simojoki M, Risteli J, Risteli L, Kauppila A. (1999). Type I and III collagen metabolites as predictors of clinical outcome in epithelial ovarian cancer. *Clin Cancer Res* 5:4091–4096.

Saudek DM, Kay J. (2003). Advanced glycation endproducts and osteoarthritis. *Curr Rheumatol Rep* 5:33–40.

Sawyers CL. (2008). The cancer biomarker problem. *Nature* 452:548–552.

Schaller S, Henriksen K, Hoegh-Andersen P, Søndergaard BC, Sumer EU, Tanko LB, Qvist P, Karsdal MA. (2005). In vitro, ex vivo, and *in vivo* methodological approaches for studying therapeutic targets of osteoporosis and degenerative joint diseases: how biomarkers can assist? *Assay Drug Dev Technol* 3:553–580.

Schetter AJ, Heegaard NH, Harris CC. (2010). Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* 31:37–49.

Schuppan D, Ruehl M, Somasundaram R, Hahn EG. (2001). Matrix as a modulator of hepatic fibrogenesis. *Semin Liver Dis* 21:351–372.

Schwartz AV, Garnero P, Hillier TA, Sellmeyer DE, Strotmeyer ES, Feingold KR, Resnick HE, Tylavsky FA, Black DM, Cummings SR, Harris TB, Bauer DC; Health, Aging, and Body Composition Study. (2009). Pentosidine and increased fracture risk in older adults with type 2 diabetes. *J Clin Endocrinol Metab* 94:2380–2386.

Senolt L, Braun M, Olejárová M, Forejtová S, Gatterová J, Pavelka K. (2005). Increased pentosidine, an advanced glycation end product, in serum and synovial fluid from patients with knee osteoarthritis and its relation with cartilage oligomeric matrix protein. *Ann Rheum Dis* 64:886–890.

Sheikh Z, Ahmad R, Sheikh N, Ali R. (2007). Enhanced recognition of reactive oxygen species damaged human serum albumin by circulating systemic lupus erythematosus autoantibodies. *Autoimmunity* 40:512–520.

Simojoki M, Santala M, Risteli J, Kauppila A. (2001). Carboxyterminal telopeptide of type I collagen (ICTP) in predicting prognosis in epithelial ovarian cancer. *Gynecol Oncol* 82:110–115.

Sjöberg JS, Bulterijs S. (2009). Characteristics, formation, and pathophysiology of glucosepane: a major protein cross-link. *Rejuvenation Res* 12:137–148.

Smith BC, Denu JM. (2009). Chemical mechanisms of histone lysine and arginine modifications. *Biochim Biophys Acta* 1789:45–57.

Somiari SB, Somiari RI, Heckman CM, Olsen CH, Jordan RM, Russell SJ, Shriver CD. (2006). Circulating MMP2 and MMP9 in breast cancer– potential role in classification of patients into low risk, high risk, benign disease and breast cancer categories. *Int J Cancer* 119:1403–1411.

Sondergaard BC, Henriksen K, Wulf H, Oestergaard S, Schurigt U, Bräuer R, Danielsen I, Christiansen C, Qvist P, Karsdal MA. (2006). Relative contribution of matrix metalloprotease and cysteine protease activities to cytokine-stimulated articular cartilage degradation. *Osteoarthr Cartil* 14:738–748.

Spickett CM, Pitt AR, Morrice N, Kolch W. (2006). Proteomic analysis of phosphorylation, oxidation and nitrosylation in signal transduction. *Biochim Biophys Acta* 1764:1823–1841.

Sternlicht MD, Werb Z. (2001). How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 17:463–516.

Strongin AY (2006). Mislocalization and unconventional functions of cellular MMPs in cancer. *Cancer Metastasis Rev* 25:87–98.

Takahashi M, Suzuki M, Kushida K, Miyamoto S, Inoue T. (1997). Relationship between pentosidine levels in serum and urine and activity in rheumatoid arthritis. *Br J Rheumatol* 36(6):637–642.

Têtu B, Brisson J, Wang CS, Lapointe H, Beaudry G, Blanchette C, Trudel D. (2006). The influence of MMP-14, TIMP-2 and MMP-2 expression on breast cancer prognosis. *Breast Cancer Res* 8:R28.

Tlsty TD, Coussens LM. (2006). Tumor stroma and regulation of cancer development. *Annu Rev Pathol* 1:119–150.

Treweek JB, Dickerson TJ, Janda KD. (2009). Drugs of abuse that mediate advanced glycation end product formation: a chemical link to disease pathology. *Acc Chem Res* 42:659–669.

Van HL, Van AE, Mareel M (2000). Collagen type I: a substrate and a signal for invasion. *Prog Mol Subcell Biol* 25:105–134.

Vlodavsky I, Abboud-Jarrous G, Elkin M, Naggi A, Casu B, Sasisekharan R, Ilan N. (2006). The impact of heparanese and heparin on cancer metastasis and angiogenesis. *Pathophysiol Haemost Thromb* 35:116–127.

Vlodavsky I, Ilan N, Naggi A, Casu B. (2007). Heparanase: structure, biological functions, and inhibition by heparin-derived mimetics of heparan sulfate. *Curr Pharm Des* 13:2057–2073.

Voorter CE, de Haard-Hoekman WA, van den Oetelaar PJ, Bloemendal H, de Jong WW. (1988). Spontaneous peptide bond cleavage in aging alpha-crystallin through a succinimide intermediate. *J Biol Chem* 263:19020–19023.

Weinberg F, Chandel NS. (2009). Mitochondrial metabolism and cancer. *Ann N Y Acad Sci* 1177:66–73.

Winding B, NicAmhlaoibh R, Misander H, Hoegh-Andersen P, Andersen TL, Holst-Hansen C, Heegaard AM, Foged NT, Brunner N, Delaisse JM. (2002). Synthetic matrix metalloproteinase inhibitors inhibit growth of established breast cancer osteolytic lesions and prolong survival in mice. *Clin Cancer Res* 8:1932–1939.

Wolf C, Rouyer N, Lutz Y, Adida C, Loriot M, Bellocq JP, Chambon P, Basset P. (1993). Stromelysin 3 belongs to a subgroup of proteinases expressed in breast carcinoma fibroblastic cells and possibly implicated in tumor progression. *Proc Natl Acad Sci USA* 90:1843–1847.

Wu CY, Wu MS, Chiang EP, Chen YJ, Chen CJ, Chi NH, Shih YT, Chen GH, Lin JT. (2007). Plasma matrix metalloproteinase-9 level is better than serum matrix metalloproteinase-9 level to predict gastric cancer evolution. *Clin Cancer Res* 13:2054–2060.

Yamamoto M, Yamaguchi T, Yamauchi M, Sugimoto T. (2009). Low serum level of the endogenous secretory receptor for advanced glycation end products (esRAGE) is a risk factor for prevalent vertebral fractures independent of bone mineral density in patients with type 2 diabetes. *Diabetes Care* 32:2263–2268.

Ylisirniö S, Höyhtyä M, Mäkitaro R, Pääakkö P, Risteli J, Kinnula VL, Turpeenniemi-Hujanen T, Jukkola A. (2001). Elevated serum levels of type I collagen degradation marker ICTP and tissue inhibitor of metalloproteinase (TIMP) 1 are associated with poor prognosis in lung cancer. *Clin Cancer Res* 7:1633–1637.

Ylisirniö S, Sassi ML, Risteli J, Turpeenniemi-Hujanen T, Jukkola A. (1999). Serum type I collagen degradation markers, ICTP and CrossLaps, are factors for poor survival in lung cancer. *Anticancer Res* 19:5577–5581.

Yoshida H, Ishiko O, Sumi T, Matsumoto Y, Ogita S. (2001). Survivin, bcl-2 and matrix metalloproteinase-2 enhance progression of clear cell- and serous-type ovarian carcinomas. *Int J Oncol* 19:537–542.

Yoshida N, Okumura K, Aso Y. (2005). High serum pentosidine concentrations are associated with increased arterial stiffness and thickness in patients with type 2 diabetes. *Metab Clin Exp* 54:345–350.

Young AL, Carter WG, Doyle HA, Mamula MJ, Aswad DW. (2001). Structural integrity of histone H2B *in vivo* requires the activity of protein L-isoaspartate O-methyltransferase, a putative protein repair enzyme. *J Biol Chem* 276:37161–37165.

Yu AE, Hewitt RE, Connor EW, Stetler-Stevenson WG. (1997). Matrix metalloproteinases. Novel targets for directed cancer therapy. *Drugs Aging* 11:229–244.

Yurchenco PD, Schittny JC. (1990). Molecular architecture of basement membranes. *Faseb J* 4:1577–1590.

Zhu GG, Risteli L, Mäkinen M, Risteli J, Kauppila A, Stenbäck F. (1995). Immunohistochemical study of type I collagen and type I pN-collagen in benign and malignant ovarian neoplasms. *Cancer* 75:1010–1017.

Corrigendum

This version contains a different figure 1 to the previous version published online as permission to use the original figure was not obtained