Novel Collagen Markers for Early Detection of Bone Metastases in Breast and Prostate Cancer Patients

Ph.d. Thesis by Diana Julie Leeming, May 2010
Technical University of Denmark, Department of Systems Biology
Nordic Bioscience
Front page pictures


2. Example of a MS/MS spectrum of a peptide from collagen type I cleaved by matrix metalloproteinase 2 (MMP-2). Fragmentation occurs and are denoted y and b ions. The fragmentation pattern reveals the amino acid sequence when analyzing using a MS database such as MASCOT.

3. Example of a competitive enzyme linked immunosorbent assay (ELISA) construction involving a biotinylated peptide (epitope-Bio) for coating of the streptavidin surface; a monoclonal antibody (mAb1); a sample and an anti-mouse labeled with horse radish peroxidase (Anti-mouse-POD).

4. Interactions between tumour cells, osteoblasts, and osteoclasts in the proximity of a bone metastasis. Tumour cells may stimulate bone cells with predominantly osteolytic or osteoblastic mediators. Increased activity of osteoblasts and osteoclasts results in the release of different biomarkers (enzymes, bone matrix components, and degradation peptides from collagen type I), which can be detected in the serum/plasma and/or urine. Adapted from [1].
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Finally I would also like to thank Dan Oersnes, my family and friends who all have supported my work.

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Diana Julie Leeming
May 2010
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase activity</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BMU</td>
<td>Bone multicellular unit</td>
</tr>
<tr>
<td>BSAP</td>
<td>Bone specific alkaline phosphatase</td>
</tr>
<tr>
<td>CAII</td>
<td>Carbonic anhydrase II</td>
</tr>
<tr>
<td>Cat K</td>
<td>Cysteine proteinase cathepsin K</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony stimulating factor 1</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CTR</td>
<td>Calcitonin Receptor</td>
</tr>
<tr>
<td>CTX-I</td>
<td>C-telopeptide of collagen type I</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine receptor 4</td>
</tr>
<tr>
<td>DPD</td>
<td>Dexitpyridinoline</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECMR</td>
<td>Extracellular matrix remodeling</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin (ET)</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Hpx</td>
<td>Hemopexin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>ICTP</td>
<td>C-telopeptide of collagen type I</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LOX</td>
<td>Lysyl oxidase</td>
</tr>
<tr>
<td>LRPS</td>
<td>Low-Density Lipoprotein Receptor-Related Protein 5</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulation factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MS</td>
<td>Mass spectroscopy</td>
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<tr>
<td>MS/MS</td>
<td>Tandem MS</td>
</tr>
<tr>
<td>MSC</td>
<td>Multipotential mesenchymal stem cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NTX</td>
<td>N-terminal telopeptide of collagen type I</td>
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<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PAD</td>
<td>Peptidylarginine deiminase</td>
</tr>
<tr>
<td>PD</td>
<td>Pyridinoline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PINP</td>
<td>Pro-peptide of collagen type I</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTH-RP</td>
<td>Parathyroid hormone-related protein</td>
</tr>
<tr>
<td>PTM</td>
<td>Post translational modifications of proteins</td>
</tr>
<tr>
<td>PY</td>
<td>Pyrrole</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>QUAD</td>
<td>Quadrupole</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor Activator for Nuclear Factor κ B</td>
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<tr>
<td>RANKL</td>
<td>Receptor Activator for Nuclear Factor κ B Ligand</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Term</td>
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<tr>
<td>-------</td>
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</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Tc99m</td>
<td>Metastable nuclear isomer of technetium-99,</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of the metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>TRAP5b</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase form of plasminogen activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</tbody>
</table>
Abstract

Bone is a common site of tumor metastasis in breast and prostate cancer patients, and occurs in more than 50% of patients with advanced cancer disease. The consequences among others are severe bone pain, pathologic bone fractures, hypocalcaemia, and spinal cord compression. The most devastating consequence is that once cancer has metastasized to bone it is incurable. According to the World Health Organization breast cancer and prostate cancer is the second most diagnosed cancer type in women and men respectively, worldwide.

At present, the gold standard for the detection of bone metastases is by the use of imaging techniques such as radiography and technetium-99 bone scans. These techniques lack sensitivity and are limited to twice a year scans. Biomarker for the assessment of protein fragments in serum and urine using the enzyme-linked immunosorbent assay (ELISA) technique may improve the ability to identify patients with early bone metastases and evaluation of treatment response. Monitoring using biomarkers of bone turnover in cancer patients could be superior to or aid traditional imaging techniques for early detection of bone metastasis. A number of markers have been presented for the evaluation of skeletal involvement in cancer patients and several have good potential. In the present work we aimed to develop novel collagen type I biomarkers that may support higher sensitivity and closer monitoring of bone metastases in combination with the existing imaging techniques and/or existing bone related markers.

First, we present a continued evaluation of a biomarker, which was developed prior to initiation of this Ph.d. This involved a bone resorption marker for young bone turnover – the so-called ALPHA C-telopeptide of collagen type I. We found that ALPHA CTX-I was specific for bone metastases and was not affected by the primary tumor in prostate cancer.

Secondly, we developed biomarkers for the N-terminal pro-peptide of collagen type I (PINP) for the human and rat species as a measure of bone formation. Monoclonal antibodies (mAb) were raised against a number of immunogenic sites in the human and rat PINP sequence. Antibodies were screened and the best were selected for further characterizations. Antibodies against corresponding epitopes in the rat and human sequence were selected and technically robust ELISAs were produced and evaluated with regards to preclinical and clinical value. It was verified that PINP indeed was assessed using these assays. The human PINP assay was evaluated for its usefulness for detection of bone metastases in a study of prostate-, lung- and breast cancer patients. This marker was highly correlated to the presence as well as the number of bone metastases and may be a useful marker for sensitive detection of bone metastases.

Finally, we combined collagen type I, the most abundant extracellular matrix protein of bone, with the proteases known to be involved in the vicious cycle of a bone metastasis. By in vitro cleavages and mass spectrometric analysis the free end of identified peptides were identified as protease-generated neo-epitopes of relevant MMPs and cathepsin K (Cat K). Unique collagen type I sequence generated by MMP-2,-9,-13 and Cat K were selected for mAb production. Antibodies were screened and characterized to be specific for the cleaved site. At present time two ELISAs have been developed from these antibody programs towards two different MMP-2, -9 and -13 generated collagen type I neo-epitopes. Both were shown to not be related to skeletal involvement in prostate-, lung- and breast cancer patients. However, one of the markers was evaluated in the rat liver fibrosis model “bile duct ligation” (BDL) due to the fact that collagen type I as well as MMP-2 and MMP-9 are known to be highly up regulated during liver fibrosis. It was seen that this marker was elevated after four weeks of BDL in rats compared to baseline and sham levels.
In conclusion, two PINP ELISAs were developed and evaluated in different preclinical and clinical studies proving that PINP was related to bone formation and bone metastases. Furthermore, two collagen type I assays against MMP generated protein fragments were developed. One assay was proven useful for the evaluation of another extracellular matrix related disease; liver fibrosis.
Sammendrag på dansk


I rapporten præsenteres først en videre evaluering af en biokemisk markør, der blev udviklet forud for dette Ph.d arbejde. Studiet involverer en knogle resorptionsmarkør for knogle re-modellering af ung knogle matrice den såkaldte ALPHA C-telopeptide af kollagen type I. ALPHA CTX-I var specifik for knoglemetastaser og ikke påvirket af den primære prostata-tumor.


Dernæst anførte vi hypotesen om at kombinere kollagen type I og proteaser, der er kendte for at være involveret i den ”onde cirkel” i knoglemetastaser, dermed er med til at nedbryde knogle matricen til små fragmenter. Kollagen type I blev kløvet med relevante matrix metalloproteinase (MMP’er) samt cathepsin K (Cat K). De dannede fragmenter, også kaldet neo-epitoper, blev identificeret ved hjælp af masse spektroskopi. Neo-epitoper genereret af MMP-2, -9, -13 og Cat K blev selekteret til antistof produktion. På nuværende tidspunkt er to ELISA assay’s blevet udviklet og evalueret med hensyn til relation til knoglemetastaser. Det viste sig, at begge assay’s ingen relation har til knoglemetastaser i bryst-, lunge- og prostatecancer patienter. Dog blev den ene markør vurderet i rotte lever fibrose modellen ”galde kanals aflukning” (BDL), da det er velkendt at kollagen type I samt MMP-2 og -9 er opregulerede i lever fibrose. Det blev her vist at et MMP-2, -9, -13 genereret kollagen type I fragment var forhøjet i uge 4 efter BDL operation i rotter sammenlignet med basis- og sham niveau.

To PINP ELISAs blev udviklet og evalueret i prekliniske og kliniske studier, der viste at markørerne er et mål for knogleformation og dermed kunne anvende til vurdering af knoglemetastaser. Derudover blev to MMP-2, -9 og -13 genererede kollagen type I ELISA assays udviklet og evaluereb. Det ene assay var anvendelig til evaluering af en anden ekstracellulær matrix relateret sygdom; lever fibrose.
Preface – Work Presented in this Ph.d Thesis

The work presented in this thesis was performed in collaboration between the Technical University of Denmark (DTU) and Nordic Bioscience A/S under the supervision of Associate Professors Vibeke Barkholt, Per Hägglund and Susanne Jacobsen (university supervisors), and Per Qvist and Inger Byrjalsen (company supervisors). The work was carried out in the period ranging from May 2007 to May 2010. The Ph.d work is presented as four original research papers, as well as two review papers. Two papers have at present time been accepted, three papers are under review, and one is presented as a draft manuscript:

1. Original paper 1 “Biochemical Markers for Monitoring Response to Therapy; Evidence for Higher Bone Specificity by a Novel Marker compared to Routine Markers” – Accepted for publication in Cancer Epidemiology Biomarkers & Prevention

2. Original paper 2 “Enzyme-linked Immunosorbent Serum Assays (ELISAs) for Rat and Human N-terminal Pro-Peptide of Collagen Type I (PINP) – Assessment of Corresponding Epitopes” – Undergoing review in Clinical Biochemistry

3. Original Paper 3 “A Newly Developed Serum N-terminal propeptide of Collagen type I assay was Associated with the number of Bone Metastases in Breast and Prostate cancer” – Undergoing review in BMC Cancer


5. Review paper 5 “Is Bone Quality Associated to Collagen Age?” – Accepted for publication in Osteoporosis International

6. Review paper 6 “Post-translational modifications of the extracellular matrix are key events in cancer progression - Opportunities for biochemical marker development” – Submitted to Cancer Epidemiology Biomarkers & Prevention
CHAPTER 1

Objectives of the present study

The aim of this project was to generate additional novel collagen type I markers that potentially could improve the detection of bone metastases.

1. The first paper presented in this Ph.d thesis had the objective to further validate the C-telopeptide marker ALPHA CTX-I, which is a bone resorption marker of newly formed bone matrix. This marker was developed prior to this thesis and was reported by our group to be a sensitive marker for detection of bone metastases [2,3].

2. The first approach for developing novel collagen type I markers included the development of a bone formation marker; N-terminal pro-peptide of collagen type I (PINP) which is released during bone formation. In humans this marker was already known to be increased in patients with bone metastases [4], however we aimed to develop PINP markers assessing corresponding epitopes in human and rat/mouse PINP sequence since no PINP assay for rodent was available at the time the PhD project was initiated (May 2007) and since no one have developed corresponding markers. Both mice and rat cancer models are used for the preclinical investigation of the pathogenesis of bone metastases such as nude mice models [5,6] and rat breast cancer model [7]. Thus a rodent PINP assay having a corresponding human PINP assay may improve the translation science for study comparisons of preclinical to clinical studies.

3. The second approach for developing novel collagen type I markers included investigations on how the combination of the main extracellular matrix of bone and proteases involved in the vicious cycle of bone metastases could be used as target for biomarker development. We hypothesized that collagen type I fragments generated by matrix metalloproteinases (MMPs) may be elevated in patients with bone metastases since MMPs are known to be produced by cancer cells [8-10] and a MMP derived epitope specific for bone would potentially be an indicator of bone metastases.
CHAPTER 2

Introduction

Bone is the most common site of tumor metastasis in cancer [11]. The incidence of bone metastases is particular common in breast and prostate cancer patients (Table 1) and arises when the primary tumor metastasizes to the bone causing a lesion where high bone remodeling occurs consequently destroying the bone structure. The consequences are often devastating symptoms such as severe bone pain, pathologic bone fractures, hypocalcaemia, spinal cord compression etc. The most devastating consequence is that once cancer has metastasized to bone it is incurable [12]. Bone metastases occur in more than 50% of patients with advanced cancer disease [12,13].

According to the World Health Organization breast cancer is the second most diagnosed cancer type in women worldwide (http://imaginis.com/breasthealth/statistics.asp#1). In 2009, an estimated 192,370 new cases of invasive breast cancer were expected to be diagnosed in women in the US alone (http://www.breastcancer.org/symptoms/understand_bc/statistics.jsp). In the US, prostate cancer holds the second highest mortality rate among men [14] and 192,280 new cases of prostate cancer were expected in 2009 (http://www.cancer.org/docroot/CRI/content/CRI_2_4_1X_What_are_the_key_statistics_for_prostate_cancer_36.asp). When a patient develops bone metastases treatment is changed radically and targeted to the bone. Bisphosphonates is one of the most commonly used drugs for these patients, which effectively inhibits osteoclasts [15]. Osteoclasts are the bone cells responsible for resorption of bone and the number, activity and survival of these cells are affected by the invasive tumor cells found in bone metastases by increasing. To initiate successful therapy it is crucial that bone metastases are detected as early as possible.

Table 1. Indicence of bone metastases in postmortem examination in different cancers. Adapted from [11].

<table>
<thead>
<tr>
<th>Primary tumor</th>
<th>Incidence of bone metastases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>73</td>
</tr>
<tr>
<td>Prostate</td>
<td>68</td>
</tr>
<tr>
<td>Thyroid</td>
<td>42</td>
</tr>
<tr>
<td>Kidney</td>
<td>35</td>
</tr>
<tr>
<td>Lung</td>
<td>36</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>5</td>
</tr>
</tbody>
</table>
State-of-the-art for the detection of bone metastases

At present, detection and assessment of treatment for bone metastases is hindered by the lack of effective and rapid methods to monitor patients. The gold standard techniques for detection of bone metastases today is by the use of imaging techniques such as radiography and Technetium-99 methyl diphosphonate bone scintigraphy ($^{99}$Tc) [16]. These methods are not suitable for close monitoring of metastases due to exposure of patients to radiation generating a significant burden for patients. Furthermore the high cost limits scans to twice a year giving patients a high risk of having undetected bone metastases. Additionally, routine radiography becomes positive only when substantial damage to the surrounding bone occurs and detection of treatment response would take months [17]. $^{99}$Tc scintigraphy measured as so-called "hot spots" (Figure 1) is used for determining the extent of metastatic disease but estimation can be time consuming, dependent on the operating person and expensive [16,18].

![Figure 1](http://emedicine.medscape.com/article/1255262-overview)
Biomarkers for the detection of bone metastases

The present work involves the use of biomarkers for improved evaluation of bone metastases in patients. Here biomarkers are considered to be the assessment of protein fragments in serum or urine using the enzyme-linked immunosorbent assay (ELISA) method, which may in part be able to describe the pathogenesis of a given disease and may be used for the evaluation of therapeutic interventions. Biomarkers may improve our ability to identify patients with early bone metastases and patients likely to respond to therapy would be useful. Monitoring of biomarkers for bone turnover in cancer patients could complement traditional imaging techniques for early detection of bone metastasis. It would be an ideal tool for close monitoring of cancer patients since biomarkers easily can be assessed in urine and/or serum at a large number of time points, due to the non-invasive nature of the sampling. The assessment of biomarkers in serum/urine is non-invasive and can be performed at a low cost. Monitoring might reveal patients developing the first bone metastasis when comparing levels to a baseline value determined at a given time point at which the patient did not have any bone metastases or biomarkers may determine the response to treatment in patients already known to have bone metastases [19]. This type of information would potentially be of high value for the cancer patients in question.

The use of existing biomarkers for bone turnover such as N-terminal and C-terminal telopeptide fragments from collagen type I, C-terminal pro-peptide of collagen type I, enzymes related to bone formation or proliferation and maturation of osteoclast has been investigated for detection of bone metastases and for monitoring of treatment response in patients with bone metastases [20-23,23-27]. Collagenous markers seem to be the most sensitive for bone metastases [2,23,25-27] but it has not yet been shown if any of the markers are superior to imaging techniques and whether biomarkers should be used alone or in conjunction with other markers and/or imaging techniques for increased detection. Therefore it is of great importance to develop novel sensitive markers for the detection of bone metastases as early as possible in breast and prostate cancer patients and to determine how these could be used in conjunction.

In the following the background of the topics important for this PhD thesis are briefly described to provide a good knowledge base for the work performed in this thesis. Please see the paper “An update on biomarkers of bone turnover and their utility in biomedical research and clinical practice.” by Leeming DJ et al. in appendix 1 for more a general update on the use of biomarkers for the evaluation of bone turnover [28].
I. Bone – Anatomy, Extracellular Matrix, Cells

Bone anatomy

The skeleton of vertebrates is formed by bone, which is a highly specialized tissue that is very effective for mechanical and homeostasis purposes. The bones have three main functions: 1) providing mechanical support for the whole body and acting as muscle attachment for locomotion, 2) acting as protective shields for the vital organs and the bone marrow, 3) providing a storage facility for ions, mainly calcium and phosphate, and for growth factors and cytokines [29,30]. Based on anatomy bones are divided into two subtypes a) The flat bones, such as the skull, the scapula, and the mandible, b) the long and short bones (tubeshaped), which include the femur, the tibia, the vertebrae, and the ribs [31]. A long bone consists of a long cylindrical tube called the diaphysis that widens into the metaphysis and the epiphysis, which are separated by the growth plate (Figure 2) [31]. The diaphysis primarily consists of cortical bone, a highly calcified (80-90%) very dense layer of compact bone, whereas both the metaphysis and the epiphysis also consist of trabecular (or cancellous) bone, a less calcified (15-25%) network of sponge like structures[31].

Figure 2. Schematic illustration of a long bone, showing the diaphysis, which consists mainly of cortical bone. The metaphyses, and the epiphyses, which also contain trabecular bone are shown, separated by the growth plates [30].
The human skeleton contains approximately 80% cortical bone and 20% trabecular bone; however, the different bones have varying ratio of cortical/trabecular bone [31]. The cortical bone is mainly required for the mechanical properties of bones, whereas the trabecular bone mainly serves metabolic purposes, although it also provides additional strength to the cortical bones [32]. These properties are reflected in the remodeling rates of these bones since the rate of remodeling is higher in trabecular bone than in cortical bone. It has been suggested that the reason for this may be assigned to the metabolic purposes of the trabecular bone [33].

Bone structure and extracellular matrix
The extracellular matrix of healthy cortical and trabecular bone mainly consists of lamellar layers. In cortical bone the lamellar bone forms concentric lamellae that surround the blood vessels—the so called Haversian system or osteons (Figure 3) [31,34,35]. The Haversian system includes a central canal with blood vessels for nutrient supply; osteocytes with tiny “hairs” that reach out for nutrients, lacunae for osteocytes and other bone cells and the concentric lamellae. Examples of lamellar bone structure in healthy structured bone visualized by polarized light is seen in figure 4. In the case of bone metastases and fracture healing the local high bone turnover affects the bone structure and the bone is not able to transfer the woven bone into the well arranged lamellar structure. Woven bone is a less organized structure synthesized initially by the bone-forming osteoblasts [36]. Woven bone structure adjacent to invading tumor cells visualized by polarized light has been shown by our group and is seen in figure 5 [3]. More details about the role of tumor cells in bone turnover in bone metastases are specified in section II.

Figure 3. Schematic overview of the Haversian system or osteon. Blood vessel, osteon, osteocyte lacuna and concentric lamella compartments are seen. Adapted from http://www.dmacc.edu/instructors/rbwollaston/Chapter_6_Skeletal_System.htm
Figure 4. Lamellar structure of bone. Left panel: Healthy trabecular bone (Henriksen H unpublished data). Right panel: Healthy bone in a breast cancer bone metastasis biopsy distant from the tumor invasion [3]. Histological images taken using polarized light.

Figure 5. Wowen and lamellar structure of bone in a breast cancer bone metastasis biopsy adjacent to tumor invasion [3]. Histological image taken using A) polarized light, B) non-polarized light. The brown stainings indicates presence of the C-telopeptide of collagen type I (ALPHA CTX-I) epitope, which is a marker of bone resorption [3].

Bone matrix can be divided into an organic and an inorganic phase, where the organic phase mainly consists of collagen type I and inorganic calcium [30]. Besides collagen type I a number of non-collagenous proteins exist in the organic phase. The non-collagenous proteins consist of 25% exogenous proteins, such as albumin and α2-HS glycoprotein, which originate from the serum, and bind to hydroxyapatite due to their acidic properties [37]. The exogenous non-collagenous proteins are hypothesized to regulate bone turnover, however this process is not well-understood [37,38]. The remaining 75% are endogenous bone-derived proteins, such as osteopontin, osteocalcin, osteonectin, alkaline phosphatase and many others, with more or less well-described roles in bone physiology [37,38].
Novel Collagen Markers for Early Detection of Bone Metastases

Collagen type I

Collagen is the main protein in the extracellular matrix of connective tissues. They are present in most tissues and are responsible for maintaining the structural integrity of vertebrates and many other multicellular organisms [39,40]. Collagens are a family of proteins and collagen type I is the most abundant type of collagen. It is widely distributed in almost all connective tissues with the exception of hyaline cartilage and is the major protein in bone, skin, tendon, ligament, sclera, cornea, and blood vessels [41]. Collagen type I consist of two alpha 1 polypeptide chains and one alpha 2 chain that are incorporated in a triple helical structure to obtain maximum tensile strength. Collagens are highly resistant to proteolysis and are mainly cleaved by specialized proteases such as cathepsins and matrix metalloproteinases (MMPs). These are described further in section II. The structure of collagen type I is seen in a schematic overview in figure 6.

![Collagen Type I](image)

**Figure 6.** Structure of collagen type I. Alpha 1 (α1) and alpha 2 (α2) chains form a triple helix.

**Synthesis and fibrillation**

Collagen type I is synthesized by osteoblasts and secreted into the extracellular matrix in the form of soluble precursors as procollagens. Protein disulphide isomerase induces the formation of inter- and intrachain disulphide bonds within the C-propeptide, allowing the association between procollagen chains [42,43] (also see figure 6). Biosynthesis of a procollagen involves different posttranslational modifications that occur in the endoplasmic reticulum: peptidylproline cis-trans isomerase is required to convert the proline residues to the trans form [44], and prolyl 4-hydroxylase is required to convert proline into hydroxyproline residues [45]. The family of lysyl oxidase (LOX) contributes to the formation of hydroxylysine. Propeptides are removed by procollagen N- and C proteinases, thereby triggering spontaneous self assembly of the collagen chains into fibrils in a zipper like manner [46]. After proteolysis short telopeptides are found at each end of the helical structure [47-49]. The major extracellular function of the C-propeptides are to remain soluble during its transportation through the cell. The N-propeptides do not prevent fibril formation, but influence the fibril shape and diameter. The triple-helical structure is stabilized by several posttranslational modifications that allow intermolecular and interfibrillar
cross-links to take place between collagen fibrils as a result of the action of LOX, which catalyzes cross-linking of lysine and hydroxylysine residues.

**Amino acid composition and molecular size**

In the helical region a glycine is found on every third position to facilitate the helix structure by the repetitive Gly-X-Y triplet, where X can be any other amino acid, but it is usually a proline, and Y is often a hydroxyproline [50]. Glycine is a necessity in every third position since it is the smallest amino acid that can occupy the limited space in the center of the triple helix [39]. The N- and C-propeptides do not encompass the Gly-X-Y repeat structure since they are not helical. The triple helix is approximately 300 nm in length and 1.5 nm in diameter. The \( \alpha_1 \) chain of human type I procollagen is composed by 1464 amino acids with a molecular size of approximately 140 kDa (SwissProt P02452 at http://www.uniprot.org/uniprot/P02452). The \( \alpha_2 \) chain of human type I procollagen is comprised of 1366 amino acids with a molecular size of approximately 130 kDa (SwissProt P08123 at http://www.uniprot.org/uniprot/P08123). Thus the total molecular size of the procollagen type I is around 410 kDa. The distribution of amino acids in collagen type I for homo sapiens and other species is seen in table 3 [51]. Note that only 1069 amino acids where identified in this publication indicating that the propeptides and the signal peptide where missing for the \( \alpha_1 \) chain. From table 3 it can be observed that without any exceptions the glycine is the amino acid most abundant in both chains in all the investigated species. Prolines are also found in a large quantity.

**Table 2.** Amino acid numbers indicating the locations of the signal peptide, N-terminal propetide, C-terminal propeptide and the chain of collagen type I of the A) \( \alpha_1 \) chain[52] and B) \( \alpha_2 \) chain [53]. Adapted from http://www.uniprot.org/uniprot/P02452 and http://www.uniprot.org/uniprot/P08123.
Table 3. Amino acids distribution in collagen chains of different species. Adapted from [51].

<table>
<thead>
<tr>
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<th>Bos taurus</th>
<th>Canis lupus</th>
<th>Rattus norvegicus</th>
<th>Danio rerio</th>
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</thead>
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<tr>
<td>Name</td>
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<td>HS.a2</td>
<td>BT.a1</td>
<td>BT.a2</td>
<td>CL.a1</td>
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<td>66</td>
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<td>H</td>
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<td>15</td>
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<td>73</td>
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**Bone cells**

The main bone cells responsible for maintaining the bone matrix are briefly described here. A complete description is however not within the scope of this thesis.

**Osteoclasts**

Osteoclasts are large multinuclear cells and are the only cells that are able to resorb the mineralized bone matrix [54]. The osteoclasts are derived from hematopoietic stem cells (HSCs), which differentiate into bone-resorbing osteoclasts through a series of steps leading to resorption [55]. The maturation of HSCs into osteoclasts is driven forward by a number of crucial steps and is highly dependent on the presence of Receptor Activator for Nuclear Factor κ B (RANK), the cytokine macrophage stimulating factor (MCS-F) as well as the regulator osteoprotegerin (OPG) [56]. MCS-F induces expression of RANK, which is the receptor for the cytokine Receptor Activator of Nuclear Factor κ B Ligand (RANKL) [57-59]. RANKL is a member of the Tumor Necrosis Factor (TNF) ligand family. Binding of RANKL leads to activation of intracellular pathways that are required for correct osteoclastogenesis. Osteoprotegerin (OPG) is a soluble receptor for RANKL involved in the regulation of both the number and the activity of the osteoclasts [60,61], and the correct balance between OPG and RANKL is crucial for maintenance of a healthy skeleton [62].
Mature osteoclasts are characterized by high expression of a series of osteoclast markers, of which TRACP, Matrix Metalloproteinase 9 (MMP-9), Cathepsin K (Cat K), carbonic anhydrase II (CAII), the a3 subunit of the V-ATPase, CIC-7, OSTM1, and the Calcitonin Receptor (CTR) are prominent and have relatively well-characterized functions in the osteoclasts [63-70].

The hallmark of a resorbing osteoclast is the formation of a ruffled border, which is an intensely convoluted membrane present inside the sealing zone [54]. Acidification occurs under the ruffled border of osteoclast leading to dissolution of the inorganic matrix of the bones. The breakdown of the collagen fibers in the organic phase is mainly mediated by the cysteine proteinase Cat K, which is highly active toward collagen at the low pH present in the resorption lacuna [71]. In addition to Cat K, MMPs are involved in the degradation of the organic matrix of bone, however research into the exact role of the MMPs is still ongoing [72].

**Osteoblasts**

Osteoblasts are the cells responsible for bone formation, and are derived from multipotential mesenchymal stem cells (MSC), which are also the precursor for chondrocytes, adipocytes, fibroblasts, and myocytes [73,74]. The osteoblasts arise through a chain of steps, involving development of their precursors called osteoprogenitors, differentiation into pre-osteoblasts to further differentiate into mature bone forming osteoblasts [75,76]. The differentiation of the MSCs into bone forming osteoblasts is controlled by a complex interplay between a series of cytokines, hormones and their corresponding receptors, of which the Bone Morphogenetic Proteins (BMPs), Transforming Growth Factor β (TGFβ), Insulin-like Growth Factors (IGFs), Wnts and their receptor Low-Density Lipoprotein Receptor-Related Protein 5 (LRP5), and parathyroid hormone (PTH) all use extensive signal transduction machinery to exert essential functions at different levels of osteoblastogenesis [77-80].

Mature bone-forming osteoblasts are never found alone; they always exist in small bone-forming clusters, which consist of a heterogeneous mixture of bone forming osteoblasts and bone-lining cells [73]. The osteoblasts are characterized by having a large nucleus, extensive Golgi and endoplasmatic reticulum, which all indicate a high level of protein synthesis [74]. The bone-forming osteoblasts express high levels of alkaline phosphatase activity (ALP) and secrete type I collagen onto bone surfaces forming new unmineralized bone matrix called osteoid [74]. After having laid down the osteoid, the osteoblasts mineralize the newly formed bone matrix by secretion of vesicles containing sufficient concentrations of calcium and phosphate to allow crystal formation [73,75]. In addition to secretion of type I collagen, the osteoblasts also synthesize other non-collagenous matrix proteins, such as osteocalcin, osteopontin, osteonectin, and bone sialoprotein, which are required for correct mineralization of the bone matrix [74].
Osteocytes

The destiny of osteoblasts includes differentiation into osteocytes or bone-lining cells, if they do not undergo apoptosis [75]. Osteoblasts differentiate into osteocytes, while they are embedded in to the bone matrix [75]. The number of osteocytes in bones is estimated to be 10 times higher than that of osteoblasts, which is primarily due to their extensive lifespan, predicted to be around 25 years, in the face of only three months for bone forming osteoblasts [81]. The osteocytes are restricted to small lacunae in the bones and are morphologically characterized by having a high number of cytoplasmic extensions interrelating them to each other and to the osteoblasts on the bone surface, thus allowing them to survive, although they are obscured in the calcified bone matrix [74,81].

The functions of osteocytes are under investigation, however, there is evidence indicating that their primary function is mechanosensation, and that the intricate network of osteocytes in the bones mediate responses to stretching and bending of the bones [81-83]. In addition to mechanosensation, there are several lines of evidence indicating that osteocytes are involved in targeted remodeling of aged bones, or bones having sustained microcracks [29,83,84].

Bone Remodeling

Remodeling occurs in order to maintain calcium homeostasis and repair microdamage thereby maintaining bone quality [29,32]. For this reason the remodeling does not occur at random sites in the skeleton but rather in a targeted manner [29]. It is a highly coordinated sequence of cellular activities, where resorption by the osteoclasts always is followed by bone formation by the osteoblasts, thereby leading to complete replacement of the removed tissue in a process referred to as coupling [85,86]. Osteoblasts and osteoclasts form the bone multicellular unit (BMU) (Figure 7) that reconstructs bone in distinct locations [87]. Bone remodeling modifies the bones, causing cortical and trabecular thickening during growth and thinning during aging. The purpose of remodeling during growth is to establish the bone strength and quality by removing damaged or aged matrix. Damage due to weariness builds up during recurring loading and needs to be replaced with new bone for reconstruction of the material composition, microarchitecture, and macroarchitecture [32].

Remodeling is initiated with recruitment of osteoclast precursors, in the combination with osteocytes and the so called bone-lining cells [32,84]. The mineralized bone matrix is then resorbed by osteoclasts, after which the reversal phase is initiated during which a cement line separating old and new bone is produced. During the reversal phase the vacated resorption pits are occupied by mononuclear cells, which prepare the resorbed area for new bone formation by the osteoblasts, which then replace the removed bone matrix [30]. The total time of a remodeling cycle is approximately 4-5 months, of which the resorption is 20-30 days, followed by 3-4 months of bone formation. In total an estimated two million remodeling sites are present in the skeleton at any given time [88]. Underlining the importance of the coupling between resorption and
formation, is the fact that imbalance in this process can lead to pathological situations, such as bone metastases [89,90] (Section II).

Figure 7. The remodeling cycle on trabecular bone. Adapted from [32].
II. Breast- and prostate cancer metastases

In 1889 Stephen Paget published a paper in the Lancet by the title of “The distribution of secondary growths in cancer of the breast” [91] describing the concept of the “seed and soil” that continues to be regarded as a major contribution to the area of cancer metastasis. The “seeds” refer to certain tumor cells with metastatic capability, and the “soil” is any organ or tissue/extracellular matrix providing a proper environment for growth of the seeds. Paget suggested that the spread of metastatic cells was organ-specific and not merely anatomic. Since Paget’s prominent publication several other investigators have further validated the complex interaction between tumor cells and the host’s organ microenvironment as one of the main determinants of metastatic spread of a given tumor. When proliferating metastatic cells invade bone they disrupt the normal bone remodeling by disturbing the coupling between osteoclasts and the bone forming osteoblasts. The "Seed and Soil" theory [8,89,91] is also known as the "vicious cycle" and involves up-regulation of the number and activity of osteoclasts and osteoblasts by the invading tumor cells [89]. In prostate cancer patients mainly osteoblastic/sclerotic bone lesion occur where the net result is increase bone formation [89,92]. Lesions in breast cancer patients are mainly osteolytic resulting in a net loss of bone [89].

Extracellular matrix remodeling

The extracellular matrix (ECM) acts as a barrier but also serves as a passive and active substrate for migrating cells. It presents signaling functions itself and acts as a protein deposit retaining numerous soluble factors; thus the ECM is associated with a large number of proteins, some of which are implicated in cancer progression and may play an important role in cancer prognosis. The ECM contains collagen type I and III, galectins, proteoglycans, such as heparansulphate 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 basement membrane, which divide organ parenchymal cells from the interstitial stroma [93]. 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 [94]. The basement membrane attaches to the epithelium on its interstitial matrix underneath and mainly consists of laminin and collagen type IV. In the case of bone metastasis collagen type I is the most abundant protein of the ECM and may be the protein which is affected the most during tumor invasion into the bone. Cross-linking of collagen with other proteins is mediated via LOX. The glycoproteins fibronectin and tenascin C modulate the integrinmediated adhesion of cells to other ECM proteins, e.g. collagen type I, and play as such a key role in cancer invasion [95].

Remodeling of the ECM is essential for maintaining normal and healthy conditions of a given tissue. In the remodeling process of normal individuals, old or damaged proteins are broken down by proteolytic activities and replaced by new, intact peptides. During pathological situations, such as cancer, fibrosis and inflammation, the balance is disturbed and tissue turnover becomes
skewed leading to an altered property of the ECM. A key factor having a great impact on the ECM remodeling are the MMPs and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [96-98]. These proteins participate not only in the breakdown of ECM components and subsequent tissue remodeling, but also assist in regulation of the microenvironment [96-98]. MMPs fulfill these functions by modulating the availability and activity of growth factors and cytokines or their receptors, and by processing adhesion and signaling receptor targets [97]. In cancer, it has been found that abnormally high levels of MMPs is associated with tumor initiation and progression in diverse tissues [97]. Although several mechanisms regulate MMP expression, the final level of control is achieved by TIMPs. Intuitively, due to their inhibitory actions, members of this family should be able to inhibit cancer invasion and thus, be anti-tumor proteins.

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 the urokinase form of plasminogen activator (uPA) produced by the endothelial cells of blood vessels surrounding the tumor; thus up-regulating both the proteolytic flow and angiogenesis [99]. During normal conditions cathepsins are controlled by their endogenous inhibitors; the cystatins superfamily of protease inhibitors [100]. Alterations 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 of tumors into surrounding tissue and vascularization [101]. The functional role of Cathepsins in tumor growth, migration, invasion, angiogenesis and metastasis has been reported in the literature [102], and 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 [101,102]. The cathepsins will not be further described here since this is out of the scope of this thesis.

Matrix metalloproteinase’s

MMPs are a large family of zinc-dependent extracellular proteases which have been sub-grouped according to their originally discovered substrates [103,104]:

- Stromelysins
- Collagenases
- Gelatinases
- Matrilysins
- Membrane-type MMPs

A detailed overview is seen in table 4.
Table 4. Sub-grouping of metalloproteinase’s. MMP-4, MMP-5, MMP-6 and MMP-22 are missing in the list since they were shown to be identical to other members [104].

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<th>Enzyme</th>
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<tr>
<td>Interstitial collagenase; Collagenase 1</td>
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</tr>
<tr>
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<td>MMP-20</td>
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<td>(B) GPI-anchored</td>
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MMPs are able to degrade almost all components of the basement membrane (BM) and the extracellular matrix (ECM) of a given tissue. A typical MMP consists of a propeptide of about 80 amino acids, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide of variable lengths (also called the hinge region) and a hemopexin (Hpx) domain of about 200 amino acids (Figure 8). Exceptions are MMP-7 (matrilysin 1), MMP-26 (matrilysin 2) and MMP-23; they lack the linker peptide and the Hpx domain and MMP-23 has a unique cysteine-rich domain and an immunoglobulin-like domain after the metalloproteinase domain. Gelatinase A (MMP-2) and gelatinase B (MMP-9), have three repeats of a fibronectin type II motif in the metalloproteinase domain. Fibronectin is a glycoprotein that binds to membrane-spanning receptor proteins, the so-called integrins [105]. In addition to integrins, fibronectin also binds ECM components such as collagen, fibrin and heparan sulfate proteoglycans. The zinc binding motif HEXXHXXGXXH in the catalytic domain, and the “cysteine switch” motif PRCGXD in the propeptide are frequent structural signatures, where three histidines and one cysteine in the respective propetides coordinate with the catalytic zinc ion. This Cys/Zn2+ coordination maintain proMMPs inactive by inhibiting a water molecule essential for catalysis from binding to the zinc atom. The threedimensional structure of the MMP domains of human proMMP1 and an active pig MMP1 are displayed in figure 9.
### Figure 8

Domain structure of MMPs. The domain organization of MMPs is as indicated: S = signal peptide; Pro = propeptide; Cat = catalytic domain; Zn = active-site zinc; Hpx = hemopexin domain; Fn, fibronectin domain; V, vitronectin insert; I = type I transmembrane domain; II = type II transmembrane domain; G = GPI anchor; Cp = cytoplasmic domain; Ca = cysteine array region; and Ig = IgG-like domain. A furin cleavage site is depicted as a black band between propeptide and catalytic domain [106].

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<td>16, 24</td>
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<td>17, 25</td>
</tr>
<tr>
<td>23</td>
</tr>
</tbody>
</table>

### Figure 9

(A) Ribbon diagram of human proMMP-1 and active pig MMP-1. The pro-domain is shown in red, catalytic domain in pink, the linker region in yellow, the hemopexin domain in green, zinc ions in purple, calcium ions in grey. The dotted circle indicates the region where the catalytic and hemopexin domains interact. Note that proMMP-1 has a larger area of contact sites than MMP-1. This results in the active form has an “open” configuration compared the “closed” configuration of proMMP-1. It is predicted that this region is where triple helical collagens binds.
MMPs - involvement in cancer proliferation

In tumors, the stringent control of MMP expression and activity is lost [107], resulting in extensive over-expression of a range of MMPs [108]. Degradation of the ECM by MMPs release several embedded molecules which inhibit apoptosis, evoke cell invasion into the tissue, support angiogenesis and modify immune responses, thus promoting cancer progression [109,110]. Embedded growth factors, such as vascular endothelial growth factors (VEGFs) and fibroblast growth factor (FGF) stimulate angiogenesis and tumor growth. The activity of MMPs is controlled by the tissue inhibitors of metalloproteinases (TIMPs). The TIMPs either directly inhibit the activity of MMPs by forming tight, non-covalent inhibitory complexes with them, or control the activation process itself [111]. In normal tissue turnover MMP expression levels are at a low level apart from periods of rapid tissue remodeling, such as wound healing processes. A tight equilibrium between MMPs and TIMPs is essential for normal tissue function; however during cancer growth and metastasis, this is disrupted.

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 [112]. 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 [113-123]. Irregular over-expression of MMPs has also been linked to metastasis of the cancer cells in cancers including those of breast [124,125], prostate [126-129], colon [130] and lung [131]. It is noteworthy that there is not a single MMP that is consistently over-expressed in all tumor type or has a regular pattern of MMP expression among the variety of human cancers [122]. The expression of MMPs in cancer tumors mirror fundamental tissue heterogeneity, since 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 over-expressed in many human tumors [132]. For example a recent immunohistochemical study of specimens from 133 patients with clinically proven prostate carcinoma using tissue arrays and specific antibodies demonstrated that MMP 1, 2, 7, 9, 11, 13, 14, and their tissue inhibitors, TIMPs 1, 2 and 3 had higher expression levels in carcinomas compared with benign pathologies [127].

The pathogenesis of the vicious cycle

Metastasis to the bone secondary from a primary breast or prostate cancer has devastating consequences for the bone microenvironment by setting up the “seed and soil” pathogenesis also known as the vicious cycle (Figure 10). The vicious cycle has been described and discussed in numerous high impact papers [6,89,133-138]. The presence of tumor cells in bone tissue affects the mechanisms of bone turnover thus influencing tissue integrity and function. The bone microenvironment is unique and provides a fertile soil for breast and other cancers to thrive. Bone is a hard but dynamic tissue that undergoes a continuous remodeling process to maintain bone.
strength. Tumor cells are able to secrete soluble factors such as hormones, cytokines and growth factors, which directly or indirectly stimulate osteoclast and osteoblast proliferation and function. The main mechanism by which bone metastases evoke dysregulation of bone turnover is perturbation of the RANKL/OPG ratio. This can be accomplished either by stimulating the secretion of RANKL in osteoblastic stromal cells via release of parathyroid hormone-related protein (PTHrP)[13,139] or by direct stimulation of osteoclasts by secretion of tumour necrosis factor alpha (TNF-α), colony stimulating factor 1 (CSF-1), Interleukin-1 (IL-1), IL-6, IL-8, IL-10 and RANKL [89]. RANKL activates its specific receptor RANK on osteoclast precursors and promotes cellular maturation in the presence of macrophage colony stimulation factor (M-CSF).

![Figure 10](image_url)

**Figure 10.** The vicious cycle of bone metastases. Factors, such as MMPs, chemokine receptor 4 (CXCR4), vascular endothelial growth factor (VEGF), and connective tissue growth factor (CTGF), target metastatic tumor cells to bone and facilitate survival within the bone microenvironment. Physical factors within the bone microenvironment, including hypoxia, acidic pH, and extracellular Ca²⁺, and bone-derived growth factors, such as TGF-β and IGFs, activate tumor expression of osteoblast-stimulatory factors, including vascular endothelial growth factor, platelet-derived growth factor (PDGF), and endothelin-1 (ET-1). Osteoclast-stimulatory factors, including RANKL, PTHrP, TGF-β, and IL-11, can also be increased. These factors stimulate bone cells, which in turn release factors that promote tumor growth in bone [137].

The ultimate outcome is an increased survival rate and number of mature osteoclasts and thus generation of an osteolytic metastasis, involving collagen type I degradation. Thus excessive bone resorption or formation occurs. During osteoclastic resorption growth factors such as bone morphogenetic protein 2 (BMP-2) and transforming growth factor beta (TGF-β) are released from the bone matrix that can stimulate tumor cells and in this manner setting up the vicious cycle.
Moreover it activates epithelial-mesenchymal transition and tumor cell invasion, increases angiogenesis and induces immunosuppression [140]. Less is known about osteoblastic metastases that also are linked to increased bone turnover, which in contrast to that in osteolytic lesion, is dominated by bone formation. Thus in this case tumor cells stimulate osteoblast function via endothelin (ET), bone morphogenetic proteins (BMPs), and insulin-like growth factors (IGFs). The manifestation of bone metastases occurs in 70 % of patients with advanced breast and prostate cancer [12]. Breast cancer metastases are predominately osteolytic (70-85%)[141] whereas the majority of prostate cancer metastases are osteoblastic (65%)[9] although mixed lesions exists in both cancer types [142].

MMPs promote proliferation of metastases through mechanisms of angiogenesis, invasion, migration, and finally colonization of the metastatic site as described earlier. However, the broad range of MMPs and their ever-present nature have made it complicated to put forward a clear definition of their involvement [12]. Clinical and experimental evidence support a role for the MMPs to promote osteoclastic bone resorption and bone metastases. The precise molecular mechanisms is unclear [112], but it has emerged that it may involve proteolytic cleavage of substrates and succeeding activation of pro-metastatic factors—such as TGF-β, IGFs, and vascular endothelial growth factors (VEGFs)—and ultimate activation of the RANKL pathway.
III. Biomarkers for the evaluation of bone metastases

An overview of collagen type I biomarkers that can be used for studying the ECM remodeling is seen in appendix 1 and in [40]. A number of groups have investigated the use of various bone-related biomarkers for the detection of bone metastases in breast or prostate cancer patients. Jung and co-workers [20] investigated ten different serum bone turnover markers for diagnosis and prediction of metastatic spread in prostate cancer patients. They showed that one resorption marker and two osteoclastogenesis markers were elevated in patients with bone metastases OPG had the best discriminating power. The same was observed for OPG by Brown JM. et al [21] and Lipton A. et al. [22]. The bone formation marker “bone specific alkaline phosphatase” (BSAP) has in a similar manner been used for the evaluation of skeletal involvement in both breast and prostate cancer patients with documented bone metastases, where BSAP correlated to number of bone metastases. Nevertheless, the correlation was strongest in prostate cancer patients due to the sclerotic nature of prostate cancer bone metastasis [23,24]. Others have found resorption markers such as the N-terminal telopeptide of collagen type I (NTX) and the C-telopeptide of collagen type I (BETA CTX-I) to be some of the best prognostic markers in metastatic breast cancer patients [23,25-27] but information about whether these markers are sensitive enough for detection of the first bone metastasis is lacking, and it is unclear which markers should be used together, to perform a diagnose for bone metastases as early as possible. It has been suggested that two or three markers should be assessed in the same samples from patients to increase sensitivity in conjunction with other diagnostic techniques [19].

Clinical studies have indicated that the alpha form of CTX-I (ALPHA CTX-I, resorption of newly formed collagen type I) may be more increased than the beta form CTX-I (BETA CTX-I, resorption of aged collagen type I) in both prostate and breast cancer patients with bone metastases [143,144]. Prior to the initiation of this PhD project our group had published that ALPHA CTX is correlated to the extent of disease as quantified by the Soloway score in which increasing score correlates with increasing number of bone metastases [2]. Between eight different bone biomarkers it appeared that the ALPHA CTX marker was the most sensitive marker since this biomarker increased the most in patients with bone metastases compared to those without (Figure 11) [2]. We also estimated the relative increase of ALPHA CTX in the patients having the first, second and third bone metastases, which was favorable compared to the intra-individual variation of healthy patients [3], but still longitudinal studies would be needed to determine whether bone metastases can be detected or even predicted by this biomarker. Additionally, the ALPHA CTX epitope was localized in the proximity of invading tumor cells in bone metastasis biopsies from breast cancer patients [3]. As presented in this thesis, this has now also been demonstrated to occur in prostate cancer bone metastasis biopsies [145].

Figure 11. Relative increases in bone resorption (ααCTX, ββCTX, NTX, C-telopeptide of collagen type I (ICTP), bone formation (BSAP) and osteoclastogenesis (OPG, RANKL, tartrate-resistant acid phosphatase (TRAP5b) 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. It was seen that ALPHA CTX-I (ααCTX) increased the most relative to Soloway score 0 among these eight markers. Adapted from [2].

Post-translational modifications – Neo-epitopes as biomarker targets
Post-translational modifications (PTMs) of proteins affect the composition or structure of proteins, and may thus present novel epitopes that can be selected as biochemical markers, so-called neo-epitopes [146,147]. Pathologically relevant protein modifications are not restricted to protease activity, although the sub-population of neo-epitopes generated through this mechanism may be of principal importance. Tools developed by these proposed techniques, focusing on specific monoclonal antibodies, may aid the understanding of the temporal events leading to PTM, and their role in cancer disease mechanisms. Figure 12 illustrates a handful of different types of PTMs that have been identified in biochemical marker development [148] (see also chapter 8 for more on PTMs):

A) Enzymatic cross-linking is often processed by LOX, which has been shown to promote the linearization of interstitial collagens, stiffening the tissues, and thus leads to neoplastic progression of tumor cells [149-152] (Figure 12A).
B-C) Oxidative damage to proteins is often caused by the action of reactive oxygen species (ROS) such as nitric oxide (NO) or hydrogen peroxide generated in cells by the mitochondrial respiratory chain [153] (Figure 12B).

D) Specific proteolytic activities is a prerequisite for a range of cellular functions and interactions with the ECM. These specific activities are tightly coordinated under physiological conditions, in which a detailed sequence of events locates the adequate proteolytic response to promote tissue turnover [31]. During remodeling of the ECM, the proteolytic action of proteases results in the generation of specific cleavage fragments (Figure 12D).

E) 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 a spontaneous non-enzymatic α to β amino acid isomerization [154]. 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 α-carboxyl [155] (Figure 12E). Peptides that contain amino acid isomerizations are often resistant to proteolysis [156,157].

F) Non-enzymatic glycosylation (also referred to as glycation) proceed through the so-called Maillard reaction, and leads to post-translational modification of proteins, nucleic acids and lipids [158] (Figure 12F). A common cause of glycation is increased blood glucose levels [159].

G) Citrullination or deimination is the term used for the conversion of the amino acid arginine to citrulline (Figure 12G). This modification is facilitated by peptidylarginine deiminases (PADs) [160,161]. The conversion of arginine into citrulline can have important consequences for the structure and function of proteins, since arginine is positively charged at neutral pH, whereas citrulline is uncharged. Citrullination thus increases the hydrophobicity of proteins, leading to changes in protein folding.
Protease cleaved neo-epitopes – Well established example

In this thesis I have focused on the protease-generated neo-epitopes since a major objective of the study was to develop novel biomarker assays towards fragments of the main bone ECM component collagen type I which is cleaved by proteases and known to be up-regulated during the vicious cycle. Even though many components of the ECM, as well as enzymes responsible for remodeling are present in different tissues, the combination of a specific protease and specific ECM protein may provide a unique combination that elucidates activity in a particular tissue or a specific disease mechanism. A well established example on the use of neo-epitopes for targeting a specific tissue is the previously described ALPHA CTX-I and BETA CTX-I assay that target the same epitope in collagen type I, but in two isoforms – the $\alpha$ and $\beta$ isoforms. Both have been found to be sensitive for the detection of bone metastases [2,143]. Recognition of the $\alpha$CTX-I or $\beta$CTX-I epitope using two specific sandwich assays is dependent on a number of conditions: 1) The epitope must be cleaved at a specific site by Cat K, 2) the epitope has to be cross-linked between the two lysine residues situated in each of the $\alpha$1 chains; and 3) the isomerization state between the aspartate and the glycine in the epitope should be $\alpha$-form for the ALPHA CTX-I assay and $\beta$-form for the BETA CTX-I assay [144,162] (Figure 13). These requirements for neo-epitopes provides very specific and sensitive assays that in this case are cell (Cat K), tissue (collagen type I) and age-specific (isomerization + crosslink) to osteoclast degradation of young or aged bone matrix. The cross-linking between epitopes also provides the possibility of designing a sandwich assay against cross-linked epitopes only. A sandwich assay provides a higher sensitivity and specificity since it allows one to measure a desired pool of similar fragments e.g. $\alpha\alpha$CTX-I or $\beta\beta$CTX-I pools and not the $\alpha\beta$CTX-I/$\beta\alpha$CTX-I mixed pools that also exist (Figure 14).
Figure 13. ALPHA CTX-I located in collagen type I. A) The CTX-I epitope ‘EKAHDGGR’ in the C-telopeptide of collagen type I generated by Cat K. The epitope includes a DG motif that can undergo non-enzymatic isomerization. B) The lysine (K) residues in two α1 chains can be cross-linked by pyridinoline (PD), dexpyrindoline (DPD) or pyrrole (PY). Adapted from [144].

Figure 14. Schematic overview of the bone collagen age profile measured as the ratio between ααCTX and ββCTX. The osteoclast-specific Cat K generated CTX epitope located in the C-terminal telopeptide of intact collagen type I exists in two isoforms: the α and β forms. The endogenous age profile is reflected in the isomer composition. Adapted from [163].
Identification of biomarkers

Identification of biomarkers has become increasingly sophisticated during the last two decades due to continuous improvements of technologies for protein identification as well as expansion of the proteomic information available. Large databases contain known protein sequences, domains, size, posttranslational modifications, and other information important for the understanding of protein function and imperative for selection of potential biomarker targets [164]. Software query tools enable matches of proteins for determining uniqueness of sequences, alignments, species homology and so forth [165]. Collectively, all these databases provide key links for biomarker identification and target evaluations. Of the various techniques for biomarker analysis, mass spectrometry (MS) has become the most fundamental method for protein identification and thus biomarker discovery [166]. MS provides a high sensitivity, selectivity, and fast analysis alongside the ability to interface to on-line liquid chromatographic separation methods (LC), which has made it exceptionally well-suited for multi-analyte measurements in complex biological systems. Typically, samples are separated by liquid chromatography (LC) followed by direct infusion into a mass spectrometer with an electrospray ion source. Even though several column formats can be used, the reversed phase LC is the most extensively used separation method. Samples purified off-line by chromatographic separation, 2-dimensional gel electrophoresis, or immunoprecipitation can also be analyzed by mass spectrometry [167]. For this purpose mass spectrometers with a MALDI (matrix assisted laser desorption ionization) ion source are often used. In MALDI analysis, sample aliquots are co-crystallized with a solid, UV-absorbing matrix on a stainless steel target which is inserted into the ion source of a mass spectrometer and irradiated with a laser having a wavelength absorbed by the solid matrix. MALDI applied to Time Of Flight (TOF) combines increased mass resolution with acquisition speed, making it extremely useful for profiling complex mixtures [168].

A commonly used method for protein identification involves digestion of proteins of interest by trypsin, separation by two dimensional (2D) gel electrophoresis, extraction of the peptides from the gel, and finally analysis by MALDI-TOF mass spectrometry to generate a peptide fingerprint [169]. This fingerprint can be applied to various programs to search databases for proteins matching the peptide fingerprint. Another protein identification strategy is based on LC-MS for separation and detection of peptides from digested proteins. Occasionally, fragmentation of peptides is required in the MS analysis which is referred to as tandem MS (MS/MS). The use of MS/MS is a feature present in many mass spectrometers and fragmentation is often driven by collision induced dissociation (CID). In CID fragmentation of peptide bonds is induced by collisions between protonated peptides and gas molecules in the mass spectrometer, thus generating a series of peptide fragments [170]. The fragmentation pattern is dependent on the amino acid sequence and peptide sequence information may be derived using this knowledge [171]. MS/MS spectra may be used to search protein databases in order to identify peptides and proteins.
In this thesis both MALDI TOF/TOF and LC-MS (quadrupole (QUAD)/TOF) was applied for biomarker identification of peptide fragments released from *in vitro* protease cleavage of collagen type I with MMPs and Cat K (See chapter 6).

**IV. Assay development -ELISA**

The development of Enzyme-Linked ImmunoSorbent Assays (ELISAs) has been a major part of this PhD thesis thus descriptions of the main steps utilized in the present work are found in this section. However, since ELISA development is a very technical and tedious procedure only a brief overview will be presented here.

**Selection of targets**

During the selection of targets it is essential to make use of protein databases available for determination of the best target for biomarker purposes as described in section III. Targets may also include newly identified protein fragments from protease cleavages identified by MS as presented in this thesis. Nevertheless one may evaluate targets for their uniqueness, homology between species, similarity to other targets, antigenicity, locations of all neo-epitope identifications e.g is an epitope cleaved in the middle by other proteases. After the selection of target peptides comprising desired properties the immunization strategy is planned. This is a fundamental step and should be performed in a well thought manner. This includes considerations regarding the design of peptides/immunogens/screenings peptides, species/strain, selection of immunogen, the possibility of obtaining native reactivity using a synthetic peptide. An example of a peptide design for immunization in mice is seen in table 5.
Table 5. Overview of peptides for use in immunizations and screenings in mice targeting collagen type I MMP cleaved neo-epitopes. Fragments noted here where identified by LC-MS during the present PhD thesis. Native seq. = A 10 amino acid peptide identified by MS including the protease generated site; Target seq. = six amino acids that are targeted for antibody production including a protease site; Immunogen= selected peptide coupled used for immunizations of mice; Screening seq.= peptide coupled to biotin used as a coater in ELISA for titer determination and screening of antibody producing hybridoma; De-selection seq.= peptides used for de-selecting antibodies with unwanted properties, here antibodies should not react towards peptides that have not been cleaved (elongated peptide); Characterization seq.= peptides used for characterization of screened antibodies.

<table>
<thead>
<tr>
<th>Collagen Type I</th>
<th>Species for target seq.</th>
<th>Native seq./ target seq.</th>
<th>Immunogen</th>
<th>Screening seq. (Selection)</th>
<th>De-selection seq.</th>
<th>Characterization Seq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO1_MMP2/9/13 NB103</td>
<td>Human</td>
<td>Target 100% to Human, mouse, rat, bovine, canfa CO1</td>
<td>540' LTGPSPPGPSPGP 549 /LTGPSP</td>
<td>LTGPSPPGP-GGC-OVA (used to order same un-coupled without GGC to use as standard curves)</td>
<td>GLTGSPSPGP-GGC-Biotin</td>
<td>-</td>
</tr>
<tr>
<td>CO1_MMP2/9/13 NB104</td>
<td>Human</td>
<td>Target 100% to Human, mouse, rat, bovine, canfa CO1</td>
<td>271' SGLDGAKGDA 280 /SGLDGA</td>
<td>SGLDGAKGDA-GGC-OVA (used to order same un-coupled without GGC to use as standard curves)</td>
<td>SGLDGAKGDA-GGC-Biotin</td>
<td>FSLDGAKGDA (Human, mouse CO1)</td>
</tr>
<tr>
<td>CO1_MMP2/9/13 NB105</td>
<td>Human</td>
<td>Target 100% to Human, rat, bovine, canfa CO1</td>
<td>755' GSPGKDGVRG' 764 /KDGVRG</td>
<td>OVA-CGG-GSPGKDGVRG (used to order same un-coupled without GGC to use as standard curves)</td>
<td>Biotin-CGG-GSPGKDGVRG</td>
<td>GSPGKDGVRL (Elongated peptide)</td>
</tr>
</tbody>
</table>

Development of monoclonal antibodies

A workflow for selection of targets and development of monoclonal antibodies is seen in figure 15. The immunization plan is initiated and approximately after six weeks/three immunization rounds the serum titer is determined for each mouse. Immunizations and titers are then followed every month. When immunizations reach the 6-7th immunization native reactivity should be tested in the serum of mice with high titers to select the best mouse for fusion. Native reactivity could be demonstrated using relevant native material. This could be human/rat serum, human/rat amniotic fluid, fetal calf serum, synovial fluid. Fusion is performed on the selected mouse. The spleen cells are fused with myeloma cells to produce hybridoma. The hybridoma cells are grown to become monoclonal by the so-called limited dilution method. Supernatants from monoclonal clones are screened for desired properties using the designed screening system.
Figure 15. Work flow for obtaining monoclonal antibodies against protein targets. A) Targets must be identified by histology, mass spectroscopy, tissue extracts; immunizations of mice takes around 5 months; selection of the best mice for screening for obtaining good clones. B) Calculation of number of mice needed for targeting one protein for biomarker development.

Usually screening is performed on a biotinylated version of the appropriate peptide using a horse radish peroxidase (HRP) labelled anti-mouse polyclonal antibody for detection as seen in figure 16. Screening usually includes testing reactivity against a non relevant peptide, the relevant peptide, an elongated peptide (if looking for a cleavage specific antibody), and peptides designed to exclude potential cross reactivity to undesired sequences.

Figure 16. Supernatant screening system for antibodies with desired properties. Here the mAb 1 is located in the supernatant and the sample would be peptides designed specifically for the screening in question. Bio-Epitope = biotinylated specific peptide. Anti-mouse-POD is HRP labelled anti-mouse antibody.

Hybridoma producing a monoclonal antibodies that are positive in the screening system are further subcloned and the subtype of the antibodies are determined. Preferably the antibody should be a IgG subtype (produced during second immune response) and not IgM (produced during first immune response). After subcloning the hybridoma cells of interest are passaged for collection of supernatant for characterization purposes and use in ELISA development. The most potential antibodies are now characterized in a preliminary ELISA or by the use of Western blots etc to obtain information about their reactivity pattern; native reactivity and specificity is tested.
**ELISA development - Technical optimizations**

After appropriate characterization steps the most promising monoclonal antibodies are employed for ELISA development. The main steps are illustrated in figure 17. It is of great importance to incorporate a given antibody in a preliminary assay to first roughly evaluate its performance in a non-optimized ELISA. Here a further de-selection of antibodies may be executed. The best clones are then integrated in a large technical evaluation that may take ½-2 years or even more depending on the antibody characteristics or the intended assay use.

**Figure 17.** Overview of the initial assay development steps included to obtain a robust and validated ELISA.

The following key steps are included:

i. Determine key reagents, buffers, sample volume and temperature/incubation time.

ii. Choose formulation of key reagents to ensure short term stability (storage buffer, storage at -20/+4°C).

iii. Optimize the amount of coater and antibody by performing a checkerboard for different incubation temperature and time in all buffer systems to be investigated:
   - 1 hr, 2hrs, 3hrs, 4hrs and overnight at 20°C
   - 1 hr, 2hrs, 3hr, 4hrs and overnight at 4°C

iv. Run a standard curve and native samples for each selected setting (incubation time/temperature). Determine at which time and temperature the highest sensitivity is obtained.

v. Determine the precision by assessing the intra-and inter variation of the assay using selected quality control (QC) samples.

vi. Determine linearity of the assay in relevant biological fluids and by using QC samples.
vii. Define the specifications of first assay format (T0) and initiate preclinical and clinical evaluations of the assay.

viii. Identify the weak points of the T0 test e.g. linearity, precision, sensitivity and address these issue in the optimisation round for obtaining the second version of the assay (T1).

Preclinical and clinical evaluation
Finally, a pivotal test of the technically stable assay is carried out to evaluate it ability to describe a biological phenomenon in the disease to which it was intended when selecting the given target. A preclinical and/or clinical study is used for the evaluation of relevance of the assay toward describing the pathology of the disease of interest.
CHAPTER 3

Biochemical Markers for Monitoring Response to Therapy; Evidence for Higher Bone Specificity by a Novel Marker compared to Routine Markers

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The aim of the present study was to compare a novel marker for high bone turnover with two routine markers for screening in prostate cancer patients. The markers were evaluated in two studies: 1) a cross-sectional study of 170 prostate cancer patients with local disease stratified by lymph node metastases (N0, N1) compared to controls and 2) a longitudinal study of 40 hormone refractory prostate cancer patients (HRPCA) stratified by skeletal involvement and followed during docetaxel and zoledronate treatment. Zoledronate was only administrated to +BM patients. Presence or absence of bone metastases (BM) was assessed by imaging techniques (MRI or X-ray) and Technetium-99m scintigraphy. The serum or urinary level of the alpha C-telopeptide of collagen type I (α₁CTX), prostate specific antigen (PSA) and total alkaline phosphatase (tALP) was assessed. PSA was elevated in both N0 and N1 patients compared to controls, while α₁CTX only was elevated in N1 patients. tALP exhibited no difference in any of the groups. In the treatment study, PSA and tALP decreased with treatment in both the -BM and +BM group compared to baseline values, showing similar effect of docetaxel or docetaxel/zoledronate treatment on these markers. On the contrary, α₁CTX did not decrease with docetaxel treatment in the -BM group compared to baseline while it decreased significantly with docetaxel/zoledronate treatment in the +BM group. Results suggest that α₁CTX is superior to PSA and tALP for identifying patients having a high risk of metastatic disease and for monitoring skeletal progression in +BM prostate cancer patients during treatment.
INTRODUCTION

Prostate cancer is one of the most common cancer types representing 19% of all cancers diagnosed in 2002 with 679,000 new cases in the western world [172]. Bone metastases are common in prostate cancer patients and arise when the primary tumor metastasizes to the bone causing a lesion of high bone remodeling destroying the bone structure. The consequences are devastating symptoms such as severe bone pain, pathologic bone fractures, increased mortality, hypocalcaemia, spinal cord compression etc [173, 174]. Bone metastases occur in more than 50-60% of patients with advanced cancer disease [13] and are incurable. Osteoblasts and osteoclasts are affected by the invasive tumor cells in the bone metastases causing increased number, activity and survival of these bone remodeling cells [89] a phenomenon known as the vicious cycle. Prostate cancer is characteristic mainly by sclerotic bone lesions especially in men failing androgen therapy [175].

To initiate successful therapy it is crucial that bone metastases are detected as early as possible. Early-stage prostate cancer can usually be cured by surgery or radiation therapy but this is not effective for metastatic disease [175]. Treatment is changed radically when a patient develops bone metastases by adding treatment targeting the skeleton. Bisphosphonates are some of the most used bone targeting drugs for these patients, and the drug effectively inhibits osteoclasts [15]. Soft tissue metastases are at present time normally targeted by chemotherapy or by docetaxel [175].

Biomarkers for detection of bone metastases

Bone markers are non-invasive, inexpensive and potentially serve as helpful tools for detecting bone metastases. In our lab we previously investigated the relation of eight biomarkers to the extent of skeletal involvement in a group of prostate, lung and breast cancer [2]. From this clinical study we concluded that collagenous markers were the best discriminators in general. The most sensitive marker for bone metastases was a novel marker of high bone turnover alpha CTX (ααCTX) that increased the most with increasing number of bone metastases. This marker provides an index of bone resorption of newly formed bone [2], [144], [3] by measuring the release of the non-isomerized form of the CTX epitope. The resorption of mature bone can be assessed by measuring the release of isomerized CTX (ββCTX), however this is not as sensitive for the detection of bone metastases as the ααCTX. The CTX epitope is generated by cathepsin K released by osteoclasts, thus being specific for bone resorption [176].

A characteristic of prostate cancer is the high levels of prostate-specific antigen (PSA) due to the increased numbers of prostate epithelial cells. PSA has become the routine marker for screening for prostate cancer, detection of recurrence, bone metastases and response to treatment [175].

Total alkaline phosphatase (tALP) is a bone formation marker, used as a routine marker for skeletal involvement, however recent publications have pointed to the fact that bone specific alkaline phosphatase (BSAP) is a better choice as an index of bone formation due to its higher specificity for bone [177], [20], [178].
Objective
The use of PSA and tALP as routine markers has become a topic for urologist whether these are the appropriate markers for detection of bone metastasis. Therefore, the aim of this study was to perform a head-to-head comparison between these two routine markers and the novel marker $\alpha$CTX in two different prostate cancer groups: 1) A cross-sectional study of newly diagnosed patients with clinically localized prostate cancer prior to radical prostatectomy. These patients were negative for bone metastases yet some had lymph node metastases and were stratified accordingly, 2) A prospective study of hormone refractory stage prostate cancer (HRPCA) stratified by bone metastases. All received docetaxel as palliative treatment but only patients with bone metastases were additionally administered zoledronate. Furthermore, immunohistochemistry was performed on metastatic, non-metastatic lymph nodes and bone metastases, using the $\alpha$CTX antibody, to investigate whether the epitope was detectable in such tissues.

MATERIALS AND METHODS

Patients and study design
Cross-sectional study: In this prospective study a total of 170 histological confirmed clinically localized prostate cancer patients were referred to the Department of Urology and Paediatric Urology, Philipps-University Marburg between January 2004 and July 2005 for having a radical retropubic prostatectomy including regional lymphadenectomy (RRP). 68 aged matched men with benign urological disorders served as controls. Physical examination, abdominal ultrasonography and bone scanning using Technetium-99 scintigraphy together with X-ray or MRI whenever appropriate, were used for clinical staging and to verify the absence of bone metastases. Second void urine samples and serum samples were collected fasting prior to RRP and stored at -80°C. After surgery the disease was pathologically staged by the Institute of Pathology, Philipps-University Marburg according to the 2002 UICC classification [179]. The study was done in accordance with the Helsinki Declaration II and Standards of Good Clinical Practice.

Longitudinal study: A total of 40 HRPCA patients were referred to the Department of Urology and Paediatric Urology, Philipps-UniversityMarburg between December 2003 and July 2005 to receive taxan-based chemotherapy. All patients underwent bone scanning in the same manner as in the cross-sectional study to verify the presence or absence of bone metastases. Patients received four treatment cycles each lasting 4 weeks: 35 mg/m2 docetaxel in week one, two, three and no treatment in week four. Patients with bone metastases additionally received 4 mg zoledronate in week 1 of every cycle. Second void urine samples and serum samples were collected fasting at baseline and at each cycle in week 1 prior to treatment and stored at -80°C. The study was done in accordance with the Helsinki Declaration II and Standards of Good Clinical Practice and approved by an institutional review board at the Medical School, Philipps University.
Quantification of biochemical markers
Samples were stored at -80 °C until assaying. Urine samples were used for estimation of high bone turnover bone resorption by assessing the level of ααCTX by urinary ALPHA CrossLaps [144] (Nordic Bioscience, Herlev, Denmark), respectively. Urinary excretion was corrected for creatinine levels measured by an automated urine analyzer (Hitachi-912, Roche, Germany). tALP was measured in heparin plasma on a clinical chemistry analyzer (Hitachi 917, Roche Diagnostics, Mannheim, Germany). The concentration of PSA was measured in serum samples on the Elecsys 2010 analyzer (Roche Diagnostics, Mannheim, Germany). All samples were tested in a blinded manner.

Statistical Analysis
Data shown are mean ± SD, unless otherwise indicated. Baseline demographic characteristics were compared with Student’s t-test for unpaired observations. The values of ααCTX and PSA were logarithmically transformed to obtain normality. Comparison between levels of each marker in controls, patients with and without lymph node metastasis was performed by analysis of variance (ANOVA) using the General Linear Models Procedure (GLM) of the Statistical Analysis System (SAS, Cary, NC). The same statistical procedure was used for comparison of levels in patients in the longitudinal study during each treatment cycle. Differences and associations were considered statistically significant if p<0.05.

Immunohistochemistry on lymph node tissue
The analyses were performed on paraffin embedded pelvic lymph node sections from six prostate cancer patients purchased for immunohistology at Biocat GmbH, Heidelberg, Germany. Three cases were lymph node positive for prostate cancer (N1) and three cases were negative (N0). Paraffin was removed and antigen retrieval was performed by overnight incubation of the sections in a Tris/EDTA buffer pH 9 at 60°C. Sections were blocked in Tris-buffered saline (TBS) containing 0.5% casein and incubated overnight at 4°C in a moist atmosphere with primary antibody diluted in Tris-buffered saline containing 0.5% casein or control without antibody. The primary antibodies employed were monoclonal; F44 raised against the αCTX epitope (Nordic Bioscience, Herlev, Denmark) (1:45000 dilution of a 3.18 mg/ml stock); and another raised against cytokeratin against pan-cytokeratin (KL1, 1:400 diluted from a 0.13 mg/mL stock). A control for αCTX specificity was performed by overnight incubation at room temperature of F44 and 50-fold excess of antigen prior to use. After incubation the sections were thoroughly washed in TBS and incubated with secondary peroxidase labeled antibody (Mouse EnVision; DakoCytomation, Glostrup, Denmark) for 30 minutes at room temperature. Finally, the peroxidase activity was visualized using DAB+ (Sigma), and the nuclei were counterstained using Harris's acidified Hematoxylin. The lymph node sections were then dehydrated through a gradient of alcohol (70-99%) and toluene, and mounted in DPX. Pictures were taken using an Olympus BX-60 light microscope equipped with an Olympus DP71 digital camera using the "Cell A" software from Olympus.
Immunohistochemistry on bone

The analyses were performed on six iliac crest or transpedicular bone metastasis bone biopsies of the spine of metastatic prostate cancer patients from archive material from the University Hospital Hamburg-Eppendorf, Hamburg, Germany kindly provided by Günter Delling. Specimens were fixed in buffered formalin (4%), decalcified by ultrasonic sound in combination with constant temperature at 24 °C and embedded in paraffin for preparation of sections of 5 μm in thickness. Immunohistochemistry on the bone tumor was performed in the same manner as described for the lymph node immunohistochemistry. F44 was diluted 1:45000 from a 3.18 mg/mL stock. Osteoclasts were visualized by their specific TRACP activity.

RESULTS

Relation to the extent of lymph metastatic disease

Patients were stratified by the metastatic involvement of their lymph nodes: 24 patients were lymph node positive (N1) and 146 patients were lymph node negative (N0). Demographic data are shown in table 1. There was no significant difference in age between the groups. As expected, the tumor node metastases (T-stage) and Gleason score showed that patients in the N1 group had more progressed disease than in the N0 group (Table 1).

The mean levels of PSA, αCTX and tALP were stratified according to metastatic disease in the lymph nodes compared to controls as seen in figure 1. PSA increased highly significantly in both the N0 group compared to controls (p<0.001) and in the N1 group compared to the N0 group (p<0.001) (Figure 1A). αCTX was not elevated in N0 patients compared to controls (Figure 1B) while highly elevated in N1 patients compared to N0 (p<0.001). No difference could be detected in the tALP across the three groups (Figure 1C).

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td><strong>Demographic data on controls and prostate cancer patients stratified by lymph node metastases</strong></td>
</tr>
<tr>
<td>Group</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>n</td>
</tr>
<tr>
<td>Mean age (range)</td>
</tr>
<tr>
<td>T-stage (pt.)</td>
</tr>
<tr>
<td>Gleason Score (pt.)</td>
</tr>
</tbody>
</table>

PCa= Prostate cancer, N0= Lymph node negative, N1= Lymph node metastases, TNM= Tumor Node Metastasis, pct= Percentage of total number of patients
Monitoring response to therapy

Patients were stratified by the metastatic involvement of their bones: 26 patients were positive for bone metastases (+BM) and 14 patients were negative for bone metastases (-BM). Six +BM patients did not participate in follow up due to discontinuing of treatment. Ten patients were lymph node positive in the -BM group. Hormonal therapy was unchanged during the observation period. Mean age and range was 72(42-87) yr.

Relative levels of PSA, $\alpha\alpha$CTX and tALP were calculated at each visit as the percentage change of the marker level in relation to the baseline level (Figure 2). PSA (Figure 2A) demonstrated a significant decrease in both the docetaxel and docetaxel/zoledronate group at each visit. In contrast, the relative decrease in $\alpha\alpha$CTX (Figure 2B) in patients +BM receiving docetaxel/zoledronate was significantly decreased compared to baseline at all timepoints ($p<0.01$ at months 1 and 4; $p<0.05$ at months 2 and 3) indicating a response to zoledronate treatment detected by $\alpha\alpha$CTX. $\alpha\alpha$CTX was not affected by the docetaxel treatment in the -BM group. For tALP the levels were decreased significantly in the docetaxel/zoledronate +BM group at visit 2-4 ($p>0.001$ at month 2, and $p>0.01$ at months 3-4) but not after 1 month.

The absolute mean values for all three markers at each visit are seen in table 2. It was seen that $\alpha\alpha$CTX was significantly elevated in the +BM group until visit 2 subsequent to two months of docetaxel/zoledronate therapy. A similar pattern was observed for tALP except for a significant elevated level at visit 4. PSA exhibited a non-significant decrease in the +BM group compared to the -BM group at each visit.

Figure 1. Levels of A) PSA, B)$\alpha\alpha$CTX and C) tALP in prostate cancer patients (PCa) without bone metastases stratified by +N an N0 and controls. * indicate significant difference between two groups.
Table 2
Marker values on prostate cancer patients stratified by ± bone metastases and visit from the treatment study.

<table>
<thead>
<tr>
<th></th>
<th>BM</th>
<th>Visit</th>
<th></th>
<th></th>
<th></th>
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<th></th>
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</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha CTX (ng/ml)</td>
<td>-</td>
<td>0.25 (0.17-0.19)</td>
<td>0.23 (0.18-0.22)</td>
<td>0.21 (0.18-0.23)</td>
<td>0.23 (0.17-0.20)</td>
<td>0.24 (0.18-0.21)</td>
<td></td>
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<tr>
<td></td>
<td>+</td>
<td>0.89 (0.64-0.79)</td>
<td>0.43 (0.25-0.29)</td>
<td>0.35 (0.23-0.27)</td>
<td>0.31 (0.20-0.25)</td>
<td>0.33 (0.19-0.22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tALP (U/L)</td>
<td>-</td>
<td>89 (54-62)</td>
<td>76 (39-45)</td>
<td>70 (35-40)</td>
<td>70 (60-73)</td>
<td>73 (38-43)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>246 (178-221)</td>
<td>185 (142-178)</td>
<td>125 (92-115)</td>
<td>111 (78-95)</td>
<td>120 (65-76)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA (ng/ml)</td>
<td>-</td>
<td>96 (170-263)</td>
<td>120 (189-263)</td>
<td>115 (201-329)</td>
<td>56 (133-285)</td>
<td>44 (94-177)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>115 (142-212)</td>
<td>119 (141-205)</td>
<td>74 (109-179)</td>
<td>34 (57-103)</td>
<td>45 (67-111)</td>
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</tbody>
</table>

BM: Bone Metastases, SD: Standard Deviation. * indicates significant difference between PCa+BM and PCa+BM at each visit.

Figure 2. Levels of A) PSA, B) αCTX and C) tALP in prostate cancer patients with (+BM) or without (-BM) bone metastases at each visit. * indicate significant decrease in the level of each marker in patients +BM compared to -BM at each visit.

Immunolocalization of the αCTX epitope in lymph nodes

Immunohistochemistry was performed on pelvic lymph node biopsies from three prostate cancer patients with (N1) or without (N0) metastatic disease. Figure 3A-B shows staining with cytokeratin and αCTX, respectively on N0 lymph nodes. It was verified that the primary cancer had not spread to the lymph nodes due to the absence of cytokeratin staining in the tissue. In adjacent sections no staining by αCTX was observed. Cytokeratin staining was detected in the N1 lymph node confirming the presence of metastatic cancer (Figure 3C). Staining by αCTX in the N1 lymph node was observed as island-like staining in the tissue (Figure 3D). The negative control for αCTX on lymph node sections showed no staining (data not shown).
Figure 3. Immunohistochemistry on pelvic lymph nodes biopsies from three prostate cancer patients. A-B are lymph node negative, C-D are lymph positive, adjacent sections. (A+C) Pan-cytokeratin staining (KL1), (B+D) αCTX staining (F44). Magnification: x10 (A-D).

Immunolocalization of the αCTX epitope in bone metastases

Immunohistochemistry was performed on bone metastases biopsies from five prostate cancer patients to investigate whether the αCTX epitope was present. Figure 4A to D shows adjacent sections from bone invaded by prostate cancer cells. Cytokeratin staining and areas of hyperchromatic nuclei confirmed the presence of tumor cells in the bone metastases (Figure 4A). Numerous osteoclasts were revealed by TRAP staining showing abnormal high numbers of osteoclasts in the bone tumor (Figure 4B). In the proximity of tumor cells and osteoclasts, a diffuse αCTX staining was observed (Figure 4C) with intense staining at sites of high bone turnover. Control showed no staining (Figure 4D).
Figure 4. Immunohistochemistry and histology on bone tumor tissue secondary from five primary prostate cancer patients. (A) Pan-cytokeratin (CK-MNF), (B) TRAP-positive staining of osteoclasts, (C) αCTX CTX staining (F44), (D) control, (adjacent sections). Magnification: x10 (A-D).
DISCUSSION

This is the first treatment study revealing longitudinal data for the novel high bone turnover marker ααCTX compared with two routinely used markers in patients with different stages of prostate cancer. Furthermore, these are first data on ααCTX in prostate cancer patients stratified by ± lymph node metastasis supported by immunohistochemistry. We performed a head-to-head comparison between the two routine markers PSA and tALP used for determining the progression of prostate cancer and ααCTX in a cross-sectional study of prostate cancer patients stratified by lymph node metastasis as well as in a longitudinal treatment study with patients treated with docetaxel and zoledronate.

Briefly the findings were as follows: 1) tALP was not elevated in any of the groups compared to controls whereas PSA was elevated in both cancer groups compared to controls or lymph node negative. In contrast, ααCTX was significantly elevated in lymph node positive prostate cancer patients compared to lymph node negative, but not in lymph node negative patients compared to controls, 2) The percentage decrease in ααCTX and tALP in patients +BM was significant decrease when compared to baseline level however not in the -BM group. This was not the case for PSA, which decreased in both the -BM and +BM group during treatment, 3) Immunohistochemistry on lymph nodes demonstrated staining for αCTX in metastatic lymph node, 4) Immunohistochemistry on bone metastasis from prostate cancer patients revealed staining for αCTX in proximity of tumor cells and high number of osteoclasts.

Biomarkers for routine use in prostate cancer patients

Emerging evidence suggest that biochemical markers have the potential for early detection of bone metastases compared to traditional imaging techniques [2], [20], [178], [180], [14], [143], [181]. PSA is the most extensively used biomarker for determining prostate cancer stage and for following the responds to systemic treatment of metastatic disease. The general use and confidence in PSA as a marker has become controversial, as the studies has revealed high occurrence of false-negative diagnosis of prostate cancer performed from PSA assessments [182],[183]. This may also compromise the use of this marker for determining skeletal involvement.

Physiologically, it is not anticipated that PSA is specific for bone metastases. PSA is highly expressed by prostate cancer cells as well as by normal prostate epithelial cells and thus is helpful for following tumor behavior but compromises the use of this marker as indicator for bone metastases in which osteoclasts and osteoblast are the main cells involved in the vicious cycle. Total ALP has been the most often used marker for detecting increased bone formation in metastatic prostate cancer [177], being highly elevated as they mainly develop sclerotic bone lesions. tALP consist of a pool of isoforms originating from various tissues such as liver, kidney, bone and in healthy individuals only around 50% of the tALP originates from bone remodeling
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[177]. $\alpha\alpha$CTX is a highly bone specific resorption marker released during the pathology of bone metastases. Here the normal bone turnover is disassociated, and bone resorption occurs on newly formed bone. Even though the bone lesions are mainly sclerotic a high level of bone resorption is ongoing within these lesions together with the fact that many patients will have a mix of sclerotic and lytic lesion [9].

Biomarkers in lymph node metastatic patients
The data represented here demonstrate that PSA could discriminate between patients with and without metastatic disease in the lymphs, however the elevation in patients without lymph node metastasis compared to controls revealed the non-specificity for metastases. In contrast, $\alpha\alpha$CTX was more related to the metastatic disease than PSA and tALP. We speculate that patients with a high risk of having developing or hidden bone metastases by already having the first cancer cells spread to the skeleton could be identified by $\alpha\alpha$CTX.

A limited amount of information is available on markers assessed in prostate cancer patients stratified by lymph node metastases. Part of the author group has previously investigated PSA compared to a number of bone markers in which they found the same pattern for PSA [14]. However, they reported that tALP was significantly elevated in patients with localized disease and in lymph node positive patients, which was not observed in the present cohort. The reason for this discrepancy is unclear. To further elucidate the role of tALP, a study with a higher number of patients should be performed. Another group [20] additionally investigated the ability of a formation marker to diagnose and predict metastatic spread in prostate cancer patients stratified by lymph node and bone metastasis compared to nine other serum markers. They showed that bone formation markers, a bone resorption marker and two osteoclastogenesis markers were elevated in patients with bone metastases of which the osteoclastogenesis marker had the best discriminating power.

Biomarkers in bone metastatic patients
$\alpha\alpha$CTX and ALP were unaffected by the docetaxel treatment in the -BM group and simultaneously affected by the zoledronate treatment in the +BM group. However it is clearly seen that there is a trend that tALP decreases with docetaxel treatment of -BM patients whereas this is not the case for $\alpha\alpha$CTX. Additionally, it was observed that $\alpha\alpha$CTX decrease significantly after the first month of treatment of docetaxel/zoledronate in contrast to tALP, which was significantly decrease after two months of treatment. This observation is in line with knowledge that resorption measurements are more dynamic than formation measurements as well as the fact that a formation cycle follows a resorption cycle. This implies higher specificity for monitoring of the bone targeting treatment of bone metastases by $\alpha\alpha$CTX. Monitoring by PSA and tALP appeared to be influenced by the docetaxel treatment; especially for PSA the levels were similar in the two patient groups, which complicates the evaluation of skeletal involvement and response to treatment of bone.
metastases. The inclusion of control groups would have been interesting to further investigate the sensitivity by ααCTX for bone metastases however for ethical reasons such a group did not exist. ααCTX mean levels in the +BM group reached those of the -BM group at visit 2 subsequent to two months of docetaxel/zoledronate therapy indicating a normalization of the bone turnover of young bone within the bone metastases in these patients at this point. Mean levels of PSA were at the same level in both treatment groups at all visits. This suggests that PSA can be used for monitoring docetaxel treatment of the primary tumor cells without any reflection on bone turnover. Normalization was also seen in the tALP even though the percentage decrease relative to baseline was the same in the two different treatment groups.

Other groups have compared bone markers to PSA and tALP. Five bone turnover markers were compared to PSA and tALP in a prostate cancer cohort stratified by ±BM treated with zoledronate [184]. In this group none of the markers were significantly elevated at baseline in the progression group compared to patients without progression. During treatment the CTX marker for mature bone resorption (ββCTX) decreased the most among all markers. As we have assessed the ααCTX we cannot directly compare the results but still it seems that in this cohort a collagenous marker was superior to PSA and tALP. In another study, the markers TRACP 5b, MMP-2, MMP-9 were compared to PSA and tALP in a prostate group stratified by ±BM [185]. Here tALP and PSA were the only markers significantly increased in patients +BM compared to −BM. The MMP markers were not recommended for detection of bone metastases. This illustrates that not all markers are superior to PSA and tALP. The MMP derived collagenous marker C-terminal telopeptide of type I collagen (ICTP) was additionally compared to PSA and tALP in a group of prostate cancer patients and evaluated regarding the relation to number of BM [178]. Here ICTP was not well correlated to BM and not a superior marker for bone metastases compared to PSA and tALP. As ICTP is degraded by cathepsin k produced by osteoclasts it was, however, not expected to be bone specific but rather specific for MMP activity [186].

Immunohistochemical results
The lymph node negative stainings revealed no staining for cytokeratin and ααCTX as expected. Cytokeratin staining was positive in the metastatic lymph nodes showing an invasion by prostate cancer cells however the positive staining for ααCTX was not expected due to its cathepsin k specificity. Some groups have revealed that prostate and breast cancer cells are able to produce cathepsin k [187,188], which could explain why ααCTX is located in the presence of tumor cells. Nevertheless, 10 lymph node positive patients from a total of 14 were included in the -BM group in the treatment study and ααCTX did not decrease with docetaxel treatment. The docetaxel treatment should have a diminishing effect on the tumor cells that have metastasized to the lymph nodes thus should ααCTX decrease during this treatment. This suggests that the ααCTX assessed in urine measurements originates from osteoclastic bone resorption thus specific for bone.
The bone staining showing cytokeratin, TRAP and $\alpha\alpha$CTX staining within the bone metastases showed that $\alpha\alpha$CTX could be found in the proximity of tumor cells invading bone tissue as shown previously in bone metastases biopsies from breast cancer patients [3].

**Conclusion**

$\alpha\alpha$CTX was the only marker unaffected by the primary cancer and elevated in metastatic disease giving potential for identification of patients at high risk of having hidden bone metastases. In addition, the marker was unaffected by docetaxel treatment in patients without bone metastases, while decreasing with docetaxel and zoledronate treatment in patients with bone metastases suggesting specificity for bone targeting therapy. The epitope was furthermore located in the proximity of invading prostate cancer cells in the lymph's and bone.
CHAPTER 4

Enzyme-linked Immunosorbent Serum Assays (ELISAs) for Rat and Human N-terminal Pro-Peptide of Collagen Type I (PINP) –Assessment of Corresponding Epitopes

Collaboration between Leeming DJ1, Larsen DV1, Zhang C2, He Y2, Veidal SS1; Henriksen K2, Zheng Q2, Barkholt V3, Riis BJ4, Byrjalsen I1, Qvist P1 and Karsdal MA1.

Submitted to Clinical Biochemistry

1Nordic Bioscience A/S, Herlev, Denmark; 2Nordic Bioscience Beijing, Beijing, China; 3Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark; 4Nordic Bioscience Clinical Studies, Herlev, Denmark.

The present study describes two newly developed N-terminal pro-peptides of collagen type I (PINP) competitive enzyme-linked immunosorbent assays (ELISAs) which are able to measure the corresponding PINP epitopes in the rat- and human sequence during bone formation. Mouse monoclonal antibodies were raised against corresponding rat and human PINP sequences. The antibodies were characterized by native reactivity by ELISA and western blot using amniotic fluid and serum. A competitive assay was developed and technically optimized for each species. The rat assay was evaluated in the gold standard osteoporosis model using ovariectomized (OVX) rats, and in a rat liver fibrosis model using bile duct ligation (BDL). The human PINP was evaluated in a subset of osteoporosis patients from a randomized double-blinded placebo controlled ibandronate study. The established ELISA assays were suitable for the measurement of serum of PINP. In Western blot analysis both antibodies recognized a single band around 25kDa corresponding to the expected molecular size of PINP. Furthermore, a good native reactivity was observed against amniotic fluid and serum. Rat PINP showed similar patterns in the OVX rat. PINP levels increased significantly 3 weeks after OVX (p<0.01) and returned to sham levels 6 weeks after OVX. In the rat liver fibrosis model no changes in serum PINP were seen throughout the BDL period indicating that there was no liver contribution to this assay during liver fibrosis. In the postmenopausal osteoporosis study, the levels of human serum PINP were significantly decreased after treatment with ibandronate groups compared to placebo (p<0.001) as to be expected. The two corresponding PINP assays developed for the rat and human species were technically robust. Assessments in pre-clinical and clinical models of bone formation indicated both assays are specific for PINP. These bone turnover markers may aid in the evaluation of bone related diseases.
INTRODUCTION

Osteoporosis is one of the major bone-related diseases of the Western world and the associated fragility fractures in postmenopausal women remain a major healthcare burden [189]. Currently, the prevalence of osteoporosis in industrialized countries is approximately 40% in women in their 60s, and 70% in women in their 80s [189]. A key factor of the pathogenesis of osteoporosis is an imbalance between the function of two key players of bone turnover, namely the bone resorbing osteoclasts and the bone-forming osteoblasts. Estrogen deficiency arising after the menopause leads to acceleration of bone turnover, with the rate of bone resorption exceeding the rate of bone formation. This in turn leads to a net negative calcium balance and consequent demineralisation of bone [190,191]

The rate of bone resorption and formation can be estimated by immunoassays measuring the serum concentration or urinary excretion of different target molecules produced specifically during these cellular processes [148]. Over the past decade, a wide array of such immunoassays has been launched for in vitro, ex vivo, and in vivo investigations. Their systematic validation led to an appreciation of their utility in biomedical research into the pathogenesis of osteoporosis, improving clinical diagnosis, and the evaluation of novel treatment modalities[191]. The most common markers of bone resorption and formation are those measuring peptide fragments derived from collagen type I, such as the C-telopeptide of collagen type I (CTX-I) and N-telopeptide of collagen type I (NTX); N-terminal pro-peptide of collagen type I (PINP) and crosslinks of collagen type I D-pyridinolines (DPYR) [144,186,192-196].

The US Food and Drug Administration (FDA) has stated that “Additional biomarkers and additional surrogate markers are needed to guide product development” [191]. Accurate, early, prediction of the efficacy of drugs under development might not only avoid wasting pharmaceutical company funding of research, but also prevent patients being exposed to experimental drugs that are unlikely to be effective. Most drugs are tested first in animals, and if these studies indicate an acceptable risk-benefit ratio, are subsequently trialed in humans in a shift that is referred to as translational science. Thus it may be interesting to investigate whether biomarkers of efficacy in animals may also be predictive in humans. Biochemical markers detecting the same epitopes produced by both animals and humans during certain disease processes may provide such an opportunity.

We describe two new assays of the N-terminal pro-peptide of type I collagen (PINP) produced by rats and humans, using monoclonal antibodies. We present assays specifically designed to detect corresponding epitopes produced by both rats and humans (Figure 1). The monoclonal antibodies we discovered were characterized and adapted to ELISA development.
Novel Collagen Markers for Early Detection of Bone Metastases

Figure 1. Position of the corresponding rat PINP (---) and human PINP (-------) sequences within the alpha 1 chain of the N-terminal pro-peptide of collagen type I. The alignment was performed using the NLP CLUSTALW software.

MATERIALS & METHODS

Reagents

All reagents were standard high-quality chemicals from companies such as Merck (Whitehouse Station, NJ, USA) and Sigma Aldrich (St.Louis, MO, USA). The synthetic peptides used for monoclonal antibody production were purchased from the Chinese Peptide Company, Beijing, China.

Monoclonal antibodies

4-6 week old Balb/C mice were immunized subcutaneously in the abdomen with 200 μL emulsified antigen 50 μg/immunization using Freund’s incomplete adjuvant (Rat: OVA-C-PEEYVSPDAEVIG); (Human: KLH-CGG-PDGSESPTQETT). Alignment for PINP for the two species were performed using the NLP CLUSTALW software at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html. Consecutive immunizations were performed at two week intervals, until stable elevated sera titer levels were reached. At each bleeding, the serum titer was investigated and the mouse with the highest titer was selected for fusion. The selected mice were boosted intravenously with 50 μg rat or human PINP, respectively, in 100 μL 0.9% sodium chloride solution three days before isolation of the spleen for cell fusion.

Fusion and antibody screening

The fusion procedure has been described elsewhere [197]. Briefly, mouse spleen cells were fused with SP2/0 myeloma fusion partner cells. The hybridoma cells were cloned using a semi-solid medium method and plated into 96-well microtiter plates for further growth. Here limited-dilution was used to obtain monoclonal growth. Supernatants were screened using an indirect ELISA; biotinylated peptide Biotin-K-PEEYVSPDAEVIG-VEG (rat PINP) or Biotin-K-PDGSESPTQETT-QV
Novel Collagen Markers for Early Detection of Bone Metastases

(human PINP) was used as catcher peptides on streptavidin coated microtitre plates respectively. The specificities of clones were further tested towards free peptide PEEYVSPDAEVIG (rat PINP) or PDGSESPTDQETTQV (human PINP) respectively together with a non-sense peptide.

Characterization of clones

Native reactivity and peptide affinities of the monoclonal antibodies were assessed by displacement of rat or human third trimester amniotic fluid and serum in preliminary ELISA assays using 10 ng/mL biotinylated peptide coater on streptavidin coated microtitre plates and the supernatant from growing monoclonal hybridoma. Human amniotic fluid was obtained from pregnant women undergoing cesarean section in the Beijing Hospital after obtaining written consent. Rat amniotic fluid was drawn from the uterus of pregnant Wistar rats two days prior to giving birth. The isotyping of the monoclonal antibodies was performed using the Clonotyping System-HRP kit, cat.5300-05 (Southern Biotech, Birmingham, AL, USA). Human PINP clones were additionally tested for cross-reactivity toward the corresponding N-terminal pro-peptide of collagen type II (PIINP; TASGQPGPKGQKGE) and N-terminal pro-peptide of collagen type III (PIIINP; CPQPPTAPRPNG) due to high homology between PI/II/IIIINP. Western blot analysis were carried out of selected clones. Rat or human amniotic fluid was mixed with 2x denaturing loading buffer, heated and loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and run for 2 hrs at 20 mA per gel. The protein bands were blotted onto polyvinylidene fluoride (PVDF) membrane in a blotting chamber 1 hr at 60 mA per gel. The membrane was blocked in blocking buffer (5% skimmed milk in phosphate buffered saline buffer) overnight at 4°C and incubated 1 hr with hybridoma culture supernatant diluted 1:200 in blocking buffer shaking at RT. Normal mouse IgG antibody cat.015-000-003 (Jackson Immunoresearch, West Grove, PA, USA) was used as a primary antibody as control for specificity and secondary antibody alone to control the background. The membrane was incubated 1 hr shaking with secondary horse radish peroxidase (HRP) labeled antibody, cat.31432 (Thermo Scientific, Waltham, MA, USA) diluted 1:5000 in blocking buffer. Between all steps the membrane was washed 3x5 min in tris buffered saline (TBS) buffer. Bands were visualized using chemiluminescence detection kit (ECL) (Abcam, Cambridge, UK) and developed on film for 2 min (Kodak, Perkin Elmer, Waltham, MA, USA). The selected clones were purified using Protein G columns according to the instructions of the manufacturer (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK).

Rat and Human PINP ELISA Protocol

We labeled the selected monoclonal antibodies NB46-3G1 (rat PINP) and NB15-5A7 (Human PINP) with horse radish peroxidase (HRP) using the Lightning link HRP labeling kit according to the instructions of the manufacturer (Innovabioscience, Babraham, Cambridge, UK). Rat PINP competitive ELISA: A 96-well streptavidin plate was coated with biotinylated synthetic peptide Biotin-K-PEEVSPDAEVIG-VEG dissolved in assay buffer (10 mM phosphate buffered saline (PBS), 1% BSA, 0.1% Tween-20, pH 7.4) and incubated at 20°C for 30 minutes. 20 µL of peptide calibrator or sample were added to appropriate wells, followed by 100 µL of horse radish peroxidase
conjugated monoclonal antibody NB46-3G1 and incubated overnight at 20°C. Finally, 100 µL tetramethylbenzidine (TMB) (Kem-En-Tec cat.438OH) was added and the plate was incubated 15 minutes at 20 °C in the dark. All the above incubation steps included shaking at 300 rpm. After each incubation step the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The TMB reaction was stopped by adding 100 µL of stopping solution (1% HCl) and measured at 450 nm with 650 nm as the reference. A calibration curve was plotted using a 4-parametric mathematical fit model.

**Human PINP competitive ELISA:** A 96-well streptavidin plate was coated with biotinylated synthetic peptide Biotin-K-PDGSESPTDQETT-QV dissolved in assay buffer (100 mM Phosphate buffered saline (PBS), 1% BSA, 0.1% Tween-20, pH 7.4) and incubated at 20°C for 30 minutes. 20 µL of peptide calibrator or sample were added to appropriate wells, followed by 100 µL of horse radish peroxidase conjugated monoclonal antibody NB15-5A7 and incubated overnight at 20°C. Finally, 100 µL tetramethylbenzidine (TMB) (Kem-En-Tec cat.438OH) was added and the plate was incubated 15 minutes at 20 °C in the dark. All the above incubation steps included shaking at 300 rpm. After each incubation step the plate was washed five times in washing buffer (20 mM Tris, 50mM NaCl, pH 7.2). The TMB reaction was stopped by adding 100 µL of stopping solution (1% HCl) and measured at 450 nm with 650 nm as the reference. A calibration curve was plotted using a 4-parametric mathematical fit model. Potential cross reactivity for PIINP and PIIINP was checked against synthetic peptides for the corresponding sequences.

**Technical evaluation**

Linearity was determined by 2-fold dilutions of quality control (QC) samples and linearity was calculated as percentage of recovery of the 100% sample. The lower limit of detection (LDL) was determined from 21 zero samples (i.e. buffer) and calculated as the mean + 3x standard deviation. The inter- and intra-assay variation was determined by 10 independent runs of 5 QC samples, with each run consisting of two replicas of double determinations of the samples. Finally, a master calibrator was prepared for each assay using amino acid analysis quantification for exact peptide concentration using this as a calibrator of a standard curve.

**Ovariectomized Rat model**

In this preclinical study 40 female Sprague Dawley rats, 7 months of age, were stratified into 4 groups of 10 animals per group. Rats were anaesthetized and subjected to either sham or OVX. The OVX surgery was performed via an incision centrally on the lower back which provided access to both ovaries, which were removed. All procedures were approved by the Danish Animal Experiments Inspectorate. Animals were fed with a standard diet (no. 1324, Altromin, Lage, Germany). Weights of the animals were recorded weekly. Fasting blood samples were taken under CO2/O2 anaesthesia at baseline and at termination from the retro-orbital sinus of rats which had fasted for at least 14 hours on day 21, 42 and 56 post-surgery. The collected blood was left for 30 minutes at room temperature to clot, followed by centrifugation 2x at 1500 g for 10 minutes. The serum was then transferred to clean tubes and stored at -80°C until use.
Bile Duct ligated Rat model
The study has been described in details elsewhere[198]. Briefly, 17 female Sprague-Dawley rats aged 6 months were stratified into 6 groups: BDL- or sham-operated rats were sacrificed after 2, 4 or 5 weeks. Liver fibrosis was induced in anaesthetized rats by standard BDL, in which the bile duct was ligated in two places and dissected between the two ligations prior to closing the abdomen. In sham-operated rats, the abdomen was closed without BDL surgery. Fasting blood samples were taken under CO$_2$/O$_2$ anaesthesia at baseline and at termination from the retro-orbital sinus of rats which had fasted for at least the previous 14 hours. The collected blood was left for 30 minutes at room temperature to clot, followed by centrifugation 2x at 1500 $g$ for 10 minutes. The serum was then transferred to clean tubes and stored at -80°C until use.

Bisphosphonate-Treated Postmenopausal women
A subset of 67 patients in a double-blind, randomized trial (total n=240) was used for the evaluation of the human serum PINP assay in postmenopausal women treated with ibandronate or placebo. This was the subset of samples that remaining from the original study. Complete details of the study can be found elsewhere [199]. Patients gave written informed consent before entering the study, which was conducted according o the Declaration of Helsinki and was approved by the local ethics committee and the health authorities. In the current study, the subset of 67 postmenopausal women were from the three randomized groups receiving placebo (n=24), continuous 2.5 mg of ibandronate daily (n=22) or intermittent cyclical therapy (n =21) with 20 mg of ibandronate every other day for the first 24 days of each of 3 months. All patients received a daily dose of 400 IU of vitamin D and 500 mg of calcium. Serum samples were collected from patients after at least 6 hours of fasting. Time points analyzed in this study were at baseline and after 6 months of treatment. Data for the urinary C-telopeptide collagen type I bone resorption markers ($\beta$3CTX-I) and the bone formation serum marker osteocalcin (OC) previously published Riis et al. [199] were used for analyzing their correlation to the serum PINP assessed in subset analyzed the present study. Each correlation was performed using pooled data of both baseline and six months of treatment.

Statistical analyses
Data shown are mean ± SD, unless otherwise indicated. Basic demographic characteristics were compared with Student’s t-test for unpaired observations. In the rat osteoporosis model the comparison of rat serum PINP levels in OVX and sham rat at each time point was performed by analysis of variance (ANOVA) using the General Linear Models Procedure (GLM). The same statistical procedure was used for the liver fibrosis model for the comparison of the serum PINP level at termination and baseline for each time point. Finally, this statistical procedure was used for the comparison of human serum PINP in the bisphosphonate treated groups and the placebo group. Dunnett’s adjustment of the level of significance was employed to correct for multiple comparisons. Correlations between the biochemical markers were determined using Spearmans
Rho. Differences were considered statistically significant if $p<0.05$. GRAPH PAD PRISM 5 (Graph Pad Software, La Jolla, CA, USA) was used for calculations.

RESULTS

Clone selection and characterization

The NB15 clone 5A7 mAb was selected for human PINP and the NB46 clone 3G1 for rat PINP ELISA development. Both were determined to be IgG2b subtypes. From the Western blot analysis it was seen that the rat PINP mAb 3G1 recognized a band with a molecular size between 20-30 kDa in rat amniotic fluid and that the binding could be inhibited by the specific rat PINP synthetic peptide but by not a non-sense peptide (Figure 2A). Similarly, the human PINP mAb 5A7 recognized a single band around 25 kDa in human amniotic fluid and the recognition could be inhibited by the specific human PINP synthetic peptide (Figure 2B). No band was seen using the secondary antibody alone as a control. The native reaction was high for the 3G1 clones against both rat AF and rat serum (Figure 3A). An almost complete inhibition of the signal was observed using undiluted material. In a similar manner, the native reaction of 5A7 was high both against human AF and human serum (Figure 3B). Human AF gave a 75% inhibition of the signal and 40% for human serum.

Furthermore, no cross-reactivity to PIINP or PIIINP peptides was observed with the human PINP assay (Figure 3B).

Figure 2. Western blots showing the specific bands recognized by A) the rat PINP mAb NB46-3G1, NB46-3G1 + specific peptide and NB46-3G1 + non-sense peptide; B) the human PINP mAb NB15-5A7 and NB15-5A7 + specific peptide. Normal IgG was added as a primary antibody to check specificity and secondary antibody alone to control the background. A single band around 25-28 kDa was observed for both antibodies.
Figure 3. ELISA runs showing typical standard curves and native reactivity against amniotic fluid and serum. A) Rat serum PINP: Standard curve and inhibition of the competition ELISA using rat amniotic fluid (rat AF) and rat serum. Native material was run undiluted, 1:2, 1:4 as indicated (---). B) Human serum PINP: Standard curve and inhibition of the competition ELISA using human amniotic fluid (human AF) and human serum. Native material was run undiluted, 1:2, 1:4 as indicated (---). Furthermore, cross-reactivity against PIINP/PIIINP peptide was shown for the human assay. The signal is seen as the optical density at 450 nm, subtracting the background at 650nm, as a function of peptide concentration.

Technical evaluation

Typical standard curves are presented in figure 3, showing a good 4-parametric fit for the rat PINP (Figure 3A; $R^2=0.99$) and human PINP (Figure 3B; $R^2=0.99$). The lower limit of detection (LDL) for the rat PINP assay was 20 ng/mL and 12.2 ng/ml for the human PINP assay. Dilution recovery for rat- and human PINP was within ±15% (Table 1). The inter- and intra assay variation was below 13% for both PINP assays (Table 2).
### Table 1.
Percentage dilution recovery for the rat- and human PINP assays using rat serum quality control samples (RS) or human serum quality samples (HS) respectively.

<table>
<thead>
<tr>
<th>Dilution:1:4</th>
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<th>RS 2</th>
<th>RS 3</th>
<th>RS 4</th>
<th>RS 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat PINP ng/mL</td>
<td>141</td>
<td>182</td>
<td>191</td>
<td>280</td>
<td>3286</td>
</tr>
<tr>
<td>Human PINP ng/mL</td>
<td>161</td>
<td>212</td>
<td>285</td>
<td>363</td>
<td>461</td>
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</table>

<table>
<thead>
<tr>
<th>Dilution:1:8</th>
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<th>HS 2</th>
<th>HS 3</th>
<th>HS 4</th>
<th>HS 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
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<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Dilution:1:16</td>
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<td>97%</td>
<td>99%</td>
<td>84%</td>
<td>91%</td>
</tr>
<tr>
<td>Dilution:1:64</td>
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<td>105%</td>
<td>101%</td>
<td>86%</td>
<td>106%</td>
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<td>101%</td>
<td>100%</td>
<td>85%</td>
<td>98%</td>
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<table>
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<th>RS 2</th>
<th>RS 3</th>
<th>RS 4</th>
<th>RS 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat PINP ng/mL</td>
<td>141</td>
<td>182</td>
<td>191</td>
<td>280</td>
<td>386</td>
</tr>
<tr>
<td>Human PINP ng/mL</td>
<td>161</td>
<td>212</td>
<td>285</td>
<td>363</td>
<td>461</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dilution:1:4</th>
<th>HS 1</th>
<th>HS 2</th>
<th>HS 3</th>
<th>HS 4</th>
<th>HS 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
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<td>Dilution:1:8</td>
<td>94%</td>
<td>102%</td>
<td>98%</td>
<td>92%</td>
<td>105%</td>
</tr>
<tr>
<td>Dilution:1:16</td>
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<td>88%</td>
<td>101%</td>
<td>107%</td>
<td>111%</td>
</tr>
<tr>
<td>Dilution:1:64</td>
<td>88%</td>
<td>79%</td>
<td>109%</td>
<td>111%</td>
<td>125%</td>
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<tr>
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<td>90%</td>
<td>90%</td>
<td>103%</td>
<td>103%</td>
<td>114%</td>
</tr>
</tbody>
</table>

### Table 2.
Inter- and intraassay variation for the rat- and human PINP assay using rat serum quality control samples (RS) or human serum quality control samples (HS) respectively. The variation was calculated as the mean variation between ten individual determinations of each sample.

<table>
<thead>
<tr>
<th>Rat PINP Sample</th>
<th>Value (ng/mL)</th>
<th>Intra-assay Variability %</th>
<th>Inter-variability Variability %</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS1</td>
<td>98</td>
<td>13.9%</td>
<td>12.7%</td>
</tr>
<tr>
<td>RS2</td>
<td>160</td>
<td>8.1%</td>
<td>5.5%</td>
</tr>
<tr>
<td>RS3</td>
<td>394</td>
<td>18.4%</td>
<td>4.7%</td>
</tr>
<tr>
<td>Mean</td>
<td>13.5%</td>
<td>7.6%</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Human PINP Sample</th>
<th>Value (ng/mL)</th>
<th>Intra-assay Variability %</th>
<th>Inter-assay Variability %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS1</td>
<td>161</td>
<td>4.8%</td>
<td>10.9%</td>
</tr>
<tr>
<td>HS2</td>
<td>252</td>
<td>4.0%</td>
<td>11.0%</td>
</tr>
<tr>
<td>HS3</td>
<td>360</td>
<td>4.4%</td>
<td>9.1%</td>
</tr>
<tr>
<td>Mean</td>
<td>4.4%</td>
<td>10.3%</td>
<td></td>
</tr>
</tbody>
</table>
Rat serum PINP – Evaluation in the OVX rat study

Serum PINP levels were significantly elevated in OVX rats 3 weeks after OVX (p<0.01), but decreased towards sham levels 6 weeks after OVX (Figure 4A). A similar pattern was seen for the serum tALP levels (Figure 4B). tALP also increased in rats 3 weeks after OVX, however it was not statistically significantly different from sham, as was observed with PINP. tALP levels decreased towards sham levels 6 weeks after OVX, as seen for PINP.

Figure 4. Rat serum PINP (A) and tALP (B) levels in OVX rats at baseline, 3 weeks and 6 weeks after ovariectomy. Asterisks indicate statistical significance difference compared to sham (**= p<0.01). ns = non-significant difference. Results shown are mean ± standard error of the mean (SEM).
Rat serum PINP – Evaluation in the BDL study
Levels of serum PINP did not statistically change during the entire period of the BDL study in any of the groups (Figure 5). A trend was observed towards higher PINP levels at weeks 4 and 5 compared with baseline in BDL rats, but the difference was not significant.

![Figure 5. Rat serum PINP levels in the bile duct ligation (BDL) rat model for fibrosis. Serum PINP was assessed in sham operated rats at baseline (sham-B) and termination (sham-T) as well as in BDL operated rats at baseline (BDL-B) and termination (BDL-T). Termination time points were 2, 3 and 4 weeks after surgery. Asterisks indicate statistical significance difference compared to sham ns = non-significant difference. Results shown are mean ± standard error of the mean (SEM).](image)

Human serum PINP – Evaluation in ibandronate-treated postmenopausal women
The treatment groups were well-matched. There was no statistical difference between age, height, weight or BMI between the groups (Table 3). PINP levels were significantly decreased at 6 months in both the 2.5 mg/day and 20 mg/second day ibandronate-treated groups as compared to the placebo group (p<0.001) (Figure 6). The mean changes in PINP levels were -16% in the placebo group, -49% in the 2.5 mg/day group and -50% in the 20 mg/second day group. A highly statistical correlation was observed between the formation markers PINP versus osteocalcin (OC) (Figure 7A) as well as between PINP and the bone resorption marker βCTX-I (Figure 7B).
Table 3. Demographic data for the subpopulation of the clinical ibandronate study published by Riis et al.[199].

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Placebo</th>
<th>2.5 mg</th>
<th>20 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Mean age yr (±SD)</td>
<td>68.9±4.4</td>
<td>69.2±4.6</td>
<td>68.6±4.3</td>
</tr>
<tr>
<td>Mean height cm (±SD)</td>
<td>159.2±6.1</td>
<td>160.63±</td>
<td>160.4±5.9</td>
</tr>
<tr>
<td>Mean weight kg (±SD)</td>
<td>62.9±6.5</td>
<td>63.1±7.9</td>
<td>60.7±8.4</td>
</tr>
<tr>
<td>Mean BMI (±SD)</td>
<td>24.8±2.3</td>
<td>24.47±3.1</td>
<td>23.6±2.9</td>
</tr>
</tbody>
</table>

Figure 6. Human serum PINP levels in placebo (PL), 2.5 mg/day ibandronate (2.5 mg/d IB) and 20 mg/second day ibandronate (20 mg/sec d cyclic administrated IB) treated postmenopausal women calculated as percent change after 6 months of treatment compared to baseline. Asterisks indicate statistical significance difference in the treated women compared to the placebo group (***= p<0.001).
**DISCUSSION**

This is, to our knowledge, the first study to present the development of assays to evaluate bone formation by measuring levels of corresponding PINP in humans and rats using specific, monoclonal antibodies. Our main findings were: 1) Selected monoclonal antibodies for both rat- and human PINP showed specificity against fragments around a molecular size of 25 kDa and human PINP did not cross-react with corresponding PIINP and PIIINP sequences even though these have high homology; 2) There was a high reaction towards PINP in amniotic fluid and serum for the antibodies used for both assays; 3) Changes in rat serum PINP followed the same pattern as alterations in tALP in the rat OVX model, with serum PINP levels increasing significantly after 3 weeks of OVX and returned to sham levels after 6 weeks; 4) Rat serum PINP levels did not change significantly in the BDL fibrosis model 3, 4 and 5 weeks after surgery in both sham- and BDL-operated rats; 5) Human serum PINP levels were significantly decreased in the ibandronate-treated postmenopausal women as compared to placebo and correlated well to both OC and ββ-CTX-I.

The use of biomarkers in pre-clinical and clinical studies to evaluate safety and efficacy of drugs in development is being encouraged by the FDA [191]. Biochemical markers consisting of protein fragments from degradation of tissue may indicate the disease pathology, which may be useful for diagnostic and prognostic purposes. Additionally, these markers may detect changes resulting from intervention strategies and serve as surrogate markers of efficacy [200]. The use of biomarkers to assess bone formation in patients has been proven reliable in several bone-related diseases such as osteoporosis [201,202], Paget’s disease [203,204] and cancer patients with bone metastases [2,20]. Formation of bone is most often evaluated using bone-specific alkaline phosphatase [178,205,206], osteocalcin [207,208], or C-terminal pro-peptide of collagen type I.
(PICP) and PINP [195,209,210]. Circulating levels of PINP have also been demonstrated to correlate directly with histomorphometric indices of bone formation [211]. A few human PINP assays already exist, all except one based on monoclonal antibodies, and are used for clinical evaluation of bone formation [195,212] assessing either total or monomeric PINP. However there is no corresponding assay with a monoclonal antibody to assess PINP release in the rat species. Recently the first rat PINP serum assay was presented [213], but this is based on a polyclonal antibody and therefore is not as specific as a monoclonal-based assay and does not have an assay corresponding to human PINP sequence.

Selection of clones and ELISA development
It is well known that amniotic fluid contains large amounts of collagen type I pro-peptides [214]. Thus this material was selected for ELISAs and Western blot analysis. Characterization of the selected rat- and human PINP clones revealed that both monoclonal antibodies were specific against a protein with the molecular size of around 25 kDa corresponding to the size of PINP, which has been reported to be around 27 kDa when analyzed by SDS-PAGE [215]. Additionally, the reaction was very high for both the rat and human assays against AF and serum, indicating that these clones do indeed recognize PINP. Even though the human PINP sequence has a high homology to PIINP and PIIINP, we did not see any cross-reactivity to these sequences, further verifying the specificity to PINP. Technical evaluations of the competitive ELISAs we developed revealed that both assays were technically stable with dilution recovery of around ±15% and inter- and intra variation ranging from 4-13%. The highest sensitivity for the rat PINP assay was between 15-250 ng/mL and for the human PINP assay, between 60-500 ng/mL.

Evaluations of the rat serum PINP
The rat OVX model for osteoporosis indicated that the rat serum PINP assay may reflect bone formation, since the changes in PINP levels displayed a similar pattern to those of tALP. Although only 50% of tALP is derived specifically from bone and a bone-specific alkaline phosphatase biomarker [216] would have been preferred for this experiment, tALP has nevertheless previously been used for the evaluation of bone formation [20,145]. Both the tALP and PINP markers increased in OVX rats at the first measurement time point compared with sham-operated animals. However only PINP was statistically significantly elevated compared with sham and tALP was not. This could be due to the detection of other circulating ALP isoforms originating from liver (IALP), intestines (iALP) and placenta (pALP) [217]. Six weeks after OVX, both PINP and tALP were close to sham levels. These data are in agreement with previous studies reporting that rat PINP is elevated in the initial period after OVX but return to sham levels 8 weeks after OVX [218]. In the BDL model of liver fibrosis, PINP levels did not change between baseline and termination at any timepoint. This indicates that the epitope assessed in this novel assay is specific for the evaluation of bone formation without any contribution from the liver. Some contribution from that organ was expected since liver fibrosis leads to a high elevation in the expression and synthesis of collagen type I in the liver extracellular matrix, and PINP has been shown to be
elevated in liver fibrosis patients compared to healthy controls [219]. Furthermore, our group previously observed that PINP assessed with another rat PINP assay was increased in BDL operated rats at termination at weeks 2, 4 and 5 compared to baseline, although not in sham operated rats [220]. We speculate that the epitope recognized by the 3G1 clone employed in our novel assay may be destroyed by the high proteolytic activity known to occur in liver fibrosis, especially by MMPs [221], and thereby the epitope loses its antigenicity prior to entering circulation.

Evaluation of human serum PINP
In evaluating our human serum PINP assay in postmenopausal women treated with two different regimens of ibandronate or placebo, we expected bone formation to decrease in women receiving the treatment drug due to its inhibitory effect on bone resorption and bone formation [201]. Indeed it was seen that PINP levels decreased by around 50% after 6 months of treatment compared to baseline in the ibandronate-treated groups. This is in line with the 40% decrease in bone formation observed after three months of treatment with ibandronate 2.5 mg/day in the BONE study [201]. Our findings are also comparable to the 35% decrease from baseline seen in two bone formation markers, osteocalcin and bone-specific alkaline phosphatase, after 6 months of treatment with ibandronate at the 2.5 mg/day and 20 mg/second day dose in the full study [199]. Here, in the placebo group, osteocalcin levels decreased by a mean 10% from baseline whereas bone-specific alkaline phosphatase decreased by a mean 25%. These results are in line with our findings that PINP levels in the placebo group decreased by a mean 16%. Furthermore, the correlation studies performed in the present study showed a highly statistical correlation between human serum PINP and the formation marker serum OC indicating that the human PINP indeed is a true bone formation marker. The highly statistical correlation between human serum PINP and urinary ββCTX-I further supported this assumption since bone formation is known to be coupled to bone resorption [222].

In conclusion, we have developed two assays for the detection of corresponding PINP in rats and humans using specific monoclonal antibodies. These assays evaluate bone formation. We demonstrated that there was no contribution from the liver to PINP levels. Supplementary studies are needed to further evaluate the assays and determine their potential use in dynamic evaluations of bone-related diseases.
CHAPTER 5

A Newly Developed Serum N-terminal propeptide of Collagen type I assay was Associated with the number of Bone Metastases in Breast and Prostate cancer.

Collaboration between Leeming DJ¹, Koizumi M², Qvist P³, Barkholt V³, Zhang C⁴, Henriksen K¹, Byrjalsen I¹, Karsdal MA¹.

Submitted to BMC Cancer

¹Nordic Bioscience, Herlev, Denmark; ²National Institutes of Radiological Sciences, Anagawa 4-9-1, Chiba, Japan; ³Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark; ⁴Nordic Bioscience Beijing, Beijing, China

A number of biomarkers have been proven potentially useful for their ability to indicate bone metastases (BM) in cancer patients. The aim of this study was to investigate the relative utility of a newly developed N-terminal propeptide of collagen type I (PINP) for the detection of BM in cancer patients. Participants were 161 prostate, lung and breast cancer patients stratified by number of BM (Soloway score). PINP was assessed and correlated to number of BM. Additionally, the PINP marker was correlated to bone resorption of young (ALPHA CTX-I) and aged bone (BETA CTX-I); number of osteoclasts (Tartrate-resistant acid phosphatase 5b, TRACP5B) and osteoclast activity (CTX-I/TRACP5B). PINP was significantly elevated in breast-and prostate cancer patients +BM, compared to -BM (p<0.001), however not in lung cancer patients. A strong linear association was seen between PINP and the number of BMs. Significant elevation of PINP was observed at Soloway scores 1-4 (< 0BM) compared with score 0 (0 BM) (p<0.001). The correlation between bone resorption of young bone or aged bone and bone formation was highly significant in patients +BM and −BM (p<0.0001). Data suggest that PINP potentially could determine skeletal involvement in patients with breast or prostate cancer. Correlations suggested that coupling between bone resorption and bone formation was maintained in breast- and prostate cancer patients. This is the first study to show a relation between a newly developed PINP marker and number of bone metastases. Furthermore, this is the first investigation of the coupling between bone resorption and bone formation in prostate- and breast cancer patients.
INTRODUCTION

Several cancer types are able to metastasize to organs secondary to the primary tumor. Breast, prostate, and lung cancers are the primary tumors that most frequently metastasize to the skeleton. A metabolically active bone metastasis (BM) exerts profound effects in the local bone micro-environment, the most significant being the balance between bone resorption and bone formation - being either lytic or sclerotic metastasis [90]. This results in hypocalcaemia, which in turn leads to severe bone pain and lower quality of life. Early diagnosis and treatment of BM’s might mitigate these consequences [26].

Currently, BM in cancer patients is mainly diagnosed by imaging techniques such as Technetium-99 scintigraphy or x-ray[16,223]. Imaging techniques are valuable diagnostic tools. However their accuracy in early diagnosis or feasibility in ongoing close monitoring of patients is limited[223]. Even though scintigraphy can give quantitative information on skeletal “hot spots” containing BM’s, this assessment is expensive, invasive, time-consuming, and exposes cancer patients to irradiation, limiting its use for monitoring purposes [16]. Easy-to-use and accurate diagnostic tools would be valuable supplements to imaging techniques.

Since biochemical markers of bone turnover can be assessed non-invasively, they could prove clinically practical in providing additional systemic information of bone turnover. A panel of markers may be selected to assess disease stages and skeletal subtype of the metastasis, and thereby provide essential information for choice of treatment. The use of bone turnover markers to detect the presence of BM’s is extensively discussed in the literature. Numerous biomarkers of bone resorption, formation, and osteoclastogenesis have been evaluated for their ability to indicate BM in cancer patients [20,23,143,224-226] [2,22,25,27,227-229]. Some biomarkers may prove more useful than others for the evaluation of BM’s. Several studies suggest that collagenous markers may be the most reliable markers in general for the presence of BMs.

Bone is a dynamic tissue which is continuously remodeled throughout life, not only to maintain calcium homeostasis but also to repair micro-damage and thus maintain bone quality[30]. This continuous remodeling of bone involves cells that strive to achieve a coordinated and balanced resorption of old bone (osteoclasts) and those responsible for adequate formation of new bone (osteoblasts), in a local, coordinated and sequential manner referred to as coupling [85,86,230,231]. When normal coupling occurs in healthy adult bones the amount of bone formed is equal to that resorbed [85,86,230,232,233]. Uncoupling normally occurs when the balance between formation and resorption is dissociated, such as during normal skeletal growth, or in the pathogenesis of diseases such as osteopetrosis or osteoporosis [234,235], and turnover [30], in some, but not all, osteopetrotic mutations [230,236]. In postmenopausal osteoporosis there is an increase in both bone formation and bone resorption, however bone resorption exceeds bone
formation leading to a continuous negative bone balance, bone fragility and increased risk of fractures [222,237].

Until now it is has not been investigated whether coupling or uncoupling is present in various types of BM. The aim of this study was to evaluate the ability of a new bone formation marker (PINP)[238] to detect the presence of BM in patients with breast, lung or prostate cancer. Furthermore, we aimed to investigate whether coupling existed between bone formation and resorption, and the number of osteoclasts. These analyses were performed by correlating PINP data, indicating bone formation, to the well-established C-telopeptide of collagen type I bone resorption markers ααCTX-I [144] (of young bone) and ββCTX-I [192] (of aged bone), and TRACP5B as an index for osteoclast numbers [239-241]. The three latter biomarkers were previously assessed by our group and investigated in relation to number of BM, where all correlated to number of BM’s [2]. Both TRACP5B and CTX-I have previously been evaluated by others as well as useful markers for monitoring BM [2,20,143,184,229].

MATERIALS & METHODS

Patients and study design
The study design has been published previously [2]. Briefly, 90 breast cancer patients (45 +BM and 45 -BM), 30 lung cancer patients (16 +BM and 14 –BM) and 42 prostate cancer patients (25 +BM and 17 -BM) were referred to the Cancer Institute Hospital, Tokyo, Japan, between October 2002 and April 2004. All patients underwent bone scans using a radionuclide (Technetium-99m), as well as computer tomography (CT) and/or magnetic resonance imaging (MRI) to verify and quantify the presence of BMs. All patients with skeletal complications were newly diagnosed and none had received therapies known to influence bone turnover in the previous 2 years prior to entry to the study. One breast cancer patient had also been diagnosed with Paget’s disease and was excluded from the analysis.
All participants signed approved written consent and the study was performed in accordance with the Helsinki Declaration II and Standards of Good Clinical Practice. The Local Ethical Committee approved the study protocol.

Severity of metastatic bone disease (Soloway score)
The number of BM was recorded and the skeletal load was graded, as previously proposed by Soloway et al.[242]. Briefly, Soloway 0refers to patients without BM, Soloway 1 to patients with fewer than 6 BM, Soloway 2 to patients with 6-20 BM, Soloway 3 to patients with more than 20 but less than a “super scan” defined involvement of more than 75% of the ribs, vertebrae, and pelvic bones; and Soloway 4 to patients with a “super scan”.

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Quantification of bone resorption by serum PINP

Serum samples were collected from all patients and stored at -40°C until analysis. The concentration of PINP fragments was measured by the newly developed competitive ELISA assay for human N-terminal propetide of collagen type I (Nordic Bioscience, Herlev, Denmark). The assay was run using 20 μL undiluted serum samples in a one-step ELISA 1 hour at 4°C using a horse radish labeled monoclonal antibody against a 10 amino acid sequence in the N-terminal pro-peptide of collagen type I [243]. Mean intra-variation on 10 independent runs was 4.4% on double determinations [243]. Mean inter-variation on 10 independent runs was 5.4% on double determination [243]. Dilution recovery was 90-138% on 10 normal serum samples representing the entire range of the standard curve[243].

Bone resorption and number of osteoclasts

Data on the collagen type I bone resoption markers \(\beta\beta\)CTX-I [192] (assessing resorption of aged collagen type I) and \(\alpha\alpha\)CTX-I [144] (resorption of newly synthesized collagen type I) and the marker for the number of osteoclasts (TRACP5b) have been published previously by our group [2] and these data were used for correlations to the bone formation marker, PINP, detected in serum. The correlation between the CTX-I/TRACP5b ratio, and PINP, was used to indicate resorption activity per osteoclasts versus bone formation.

Statistical Analysis

The values of each of the biochemical markers were logarithmically transformed to obtain normality. Comparisons between the level of PINP in patients with different cancer types without BM was performed by analysis of variance (ANOVA) using the General Linear Models Procedure (GLM). The same statistical procedure was used for comparison of PINP levels in patients without and with BM for each cancer type. In the comparison of the PINP levels for each Soloway score against the level in patients without metastasis, the Dunnett’s adjustment of the level of significance was employed to correct for multiple comparisons. Correlations between the biochemical markers were determined using Spearman’s Rho. Differences and associations were considered statistically significant if \(p<0.05\). GRAPH PAD PRISM 5 (Graph Pad Software, La Jolla, CA, USA) was used for calculations.
RESULTS

PINP related to bone cancer type
Demographics of 161 cancer patients stratified according to cancer type and the presence or absence of BM have previously been reported [2]. There were no statistically significant differences in age and BMI between patients with or without BM. Figure 1 shows the mean values of the bone formation marker PINP in patients stratified according to cancer type and presence or absence of BM. There was a significantly increased level of serum PINP, indicating increased bone formation, in breast and prostate cancer patients with BM compared with those without BM. However, PINP was not able to determine the presence of BM in lung cancer patients.

![Figure 1](image)

**Figure 1.** Bone formation (PINP) marker levels in 161 breast, lung and prostate cancer patients stratified by cancer type and presence (+BM) or absence of bone metastasis (-BM). Results shown are geometric mean ± SEM.

PINP related to the extent of metastatic bone disease
Since the biomarker carried little value in diagnosing BM in lung cancer patients, this subpopulation was excluded from further analyses. Accordingly, data from breast and prostate cancer patients were pooled together for subsequent analyses (n=132). The demographic data for patients was stratified according to Soloway score. No linear associations were found between Soloway score and the demographic characteristics of patients [2]. Figure 2 shows associations between Soloway score and the mean PINP values. The marker indicated significant linear increases with advancing severity of the metastatic involvement of the skeletal system. PINP was highly significantly increased at all Soloway scores 1, 2, 3 and 4 compared with Soloway score 0 (ie, no BM) (p<0.001).
Figure 2. Bone formation (PINP) marker levels in 132 breast and prostate cancer patients stratified according to the extent of metastatic bone disease described by the Soloway score 0 (-BM), and 1-4 (+BM). Results shown are geometric mean ± SEM. *** = p<0.001 indicated highly significant elevated level in +BM patients compared to -BM, ns= non significant elevation in +BM patients as compared with -BM.

The coupling between bone formation and bone resorption in breast and prostate cancer patients

Patients with prostate or breast cancer were pooled and stratified by +BM and − BM for correlation calculations. Correlations were performed using both the ααCTX-I resorption marker as a measure of osteoclast resorption of newly synthesized collagen type I (Figure 3) and the ββCTX-I resorption marker as a measure of osteoclast resorption of aged synthesized collagen type I [163] (Figure 4). A significant correlation was observed for all our correlation tests (p<0.0001) in both +BM patients (Figure 3A+4A) and −BM patients (Figure 3B+4B) except for ββCTX-I/TRACP5b ratio vs PINP in patients with BM. Data indicated that bone formation, as assessed by PINP vs resorption of young or old bone (ααCTX-I or ββCTX-I); bone formation (PINP) vs number of osteoclasts (TRACP5b); resorption of young or old bone (ααCTX-I or ββCTX-I) vs number of osteoclasts (TRACP5b) and the activity of osteoclasts (ααCTX-I or ββCTX-I/TRACP5b) vs bone formation (PINP) all are associated in breast and prostate cancer patients both with or without BM. In patients with BM Spearman correlations were strongest when using the resorption marker ααCTX-I rather than ββCTX-I.
Figure 3. Correlations between $\alpha\alpha$CTX-I and PINP; $\alpha\alpha$CTX-I and TRAC5b; TRACP5b and PINP; $\alpha\alpha$CTX-I/TRACP5b ratio and PINP in patients +BM (A) and –BM (B). A significant correlation was observed for all cases. Spearman Rho correlation ($r$) is stated for each correlation in the respective figures. *** = $p<0.001$ indicated highly significant elevated levels for Soloway score 1,2,3,4 compared to score 0.

Figure 4. Correlations between $\beta\beta$CTX-I and PINP; $\beta\beta$CTX-I and TRAC5b; TRACP5b and PINP; $\beta\beta$CTX-I/TRACP5b ratio and PINP in patients +BM (A) and –BM (B). A significant correlation was observed for all cases. Spearman Rho correlation ($r$) is stated for each correlation in the respective figures.
DISCUSSION

To our knowledge this is the first study describing the levels of a newly developed PINP marker in breast, prostate and lung cancer patients with and without BM. Furthermore, to our knowledge this is the first study investigating coupling between bone resorption and bone formation related to number of osteoclasts in a clinical trial of breast and prostate cancer patients. Only one small study on coupling in prostate cancer patients has been conducted, which included administration of gonadotropin-releasing hormone agonist treatment [244]. In these patients coupling between bone resorption and formation was observed. Our main findings were: 1) Elevated levels of bone formation in prostate- and breast cancer patients with BM was found compared to patients without BM, however not in lung cancer patients; 2) Bone formation was increased according to number of BM when stratified by the Soloway score with significant increase at score 2, 3 and 4; 3) There was significant correlations between bone formation (PINP), resorption (CTX-I) and number of osteoclasts (TRACP5B) in prostate- and breast cancer patients with BM.

The occurrence of tumor cells in bone tissue have a strong impact on the mechanisms of bone turnover due to the secretion soluble factors such as hormones, cytokines and growth factors which directly or indirectly stimulate osteoclast and osteoblast proliferation and function[89,90]. This cell-cell interaction is commonly referred to as the vicious cycle. Breast cancer metastases are predominately osteolytic (70-85%)[89] whereas the majority of prostate cancer metastases are osteoblastic (65%)[141] although mixed lesions exists in both cancer types [9]. These differences provide the rationale for investigating different biomarkers in evaluating BM in patients with different types of cancer. The results may be useful for tailoring treatment for individual patients.

One of the pathways in which osteolytic BM results in dysregulation of bone turnover is through the ability of tumor cells to stimulate the secretion of RANK ligand in osteoblastic stromal cells [245]. This promotes differentiation of cells of the hematopoietic cell lineage into osteoclasts in the presence of macrophage colony stimulation factor (M-CSF), via release of parathyroid hormone-related protein (PTH-RP), as a consequence of the vicious cycle [90,133,139,246]. This stimulation of cellular activities in the local bone environment results in aggressive osteolysis, resulting in a local imbalance in the coupling between bone resorption and bone formation.

Less information is available about sclerotic metastases that, in contrast to osteolytic lesions, lead to an excess of bone formation over bone resorption. One of the main drivers in this subtype of metastasis is the stimulation of osteoblast function via endothelin (ET), bone morphogenetic proteins (BMPs), and insulin-like growth factors (IGFs).
PINP and bone formation related to bone metastases

In the present paper we evaluated a newly developed PINP marker for the detection of BM in prostate-, lung- and breast cancer patients. This human PINP monoclonal antibody marker differs from other PINP markers [195,210,212] in that it has a parallel PINP marker for the rat species. The rat marker also uses a monoclonal antibody to measure the corresponding epitope in the rat PINP sequence, and thus offers potential value in translational science between rat breast- or prostate cancer models to human studies [191,238]. We found that PINP levels, indicating bone formation, were significantly higher in patients with BM than in patients without BM in the case of prostate and breast cancers but not lung cancer.

Data stratified according to the Soloway score indicated that this novel PINP marker was correlated to the number of BM. The marker was highly significantly elevated at all Soloway scores, indicating that this marker is very sensitive for the detection of BM. However, we do not have data available to determine whether it is more sensitive than scintigraphy. Using a different assessment of the PINP marker, another group showed that PINP levels were significantly elevated in breast cancer patients with BM compared to those without BM, and correlated to the number of BM’s [247]. A PINP marker was also evaluated in 64 prostate cancer patients stratified by -BM, -BM/+lymph node metastases or +BM. Here PINP was elevated in the –BM/+lymph node metastases and +BM groups when compared to the –BM group [248]. Interestingly, PINP was even elevated 8 months prior to detection of the first BM by scintigraphy, indicating PINP is a powerful marker for very early diagnosis of BM which might not be detected by other means. Similar findings come from Koizumi et al [4] who showed several bone formation markers (PINP, C-terminal propeptide of collagen type I (PICP) and bone alkaline phosphatase (BALP) all were elevated in prostate cancer patients with BM compared to those without BM. Nevertheless, PINP was the best discriminator of all three markers of the extent of disease. In a further study, bone formation as assessed by BALP was evaluated in 323 prostate-, lung- and breast cancer patients stratified according to extent of skeletal disease (EOD) determined by x-ray. This study showed that bone formation correlated to the number of BM in the total patient population, and specifically to the subsets of prostate and breast cancer patients, but to a lesser degree in lung cancer patients [24]. These findings correspond with our present study.

PINP compared to other bone formation markers for the detection of BM

Our findings are also consistent with those of Jung et. al. [20], who analyzed 10 different serum markers and found that PINP was significantly elevated in patients with BM than in those without. Moreover, our group previously reported that the bone formation marker, bone specific alkaline phosphatase (BSAP), was also significantly elevated in patients with prostate or breast cancer and BM than in patients without –BM, but not in lung cancer patients [2]. These findings indicated that bone formation was not elevated in this set of lung cancer patients. Recently, another group also demonstrated that BSAP was elevated in prostate cancer patients +BM and correlated to the EOD, whereas the formation marker osteocalcin (OC) did not [249]. Similar data for OC have been published by Hegele A et. al.[14] showing that OC was not elevated in prostate cancer patients...
Investigations of biochemical markers of bone turnover in lung cancer patient are very limited. In contrast to the present study Kobayashi et al. [250] found that PINP was elevated in lung cancer patients +BM compared to patients –BM with 79% sensitivity. Different analytical methods may in part explain these different outcomes.

It may be difficult to directly compare different bone formation markers since they all are of a different nature. PINP is a collagenous marker assessing the released N-terminal pro-peptide of collagen type I during bone formation; OC is a non-collagenous marker found in the bone matrix and is thought to be released by osteoblasts when involved in the mineralization of bone [208,251,252]; bone alkaline phosphatase is an enzyme found on the surface of osteoblasts but its function is not clearly understood, however it is known that osteoblasts become alkaline phosphatase positive during mineralization [253]. The expression of the collagen type I gene is known to be expressed prior to the alkaline phosphatase genes and alkaline phosphatase prior to the OC genes indicating that collagen type I derived markers may be most helpful for early detection of BM [254]. Different assays assessing different epitopes of the same bone formation protein may further complicate the picture. Thus, several statements in the bone field have been made with regards to the relevance of assessing either the monomeric or trimeric form of the PINP peptide [215,255]. Further research is needed to understand why measurements of the same protein by different assessment technologies produce different outcomes, and the possible pathological rationale for the discrepancies.

There is no general consensus in the literature regarding the sensitivity of PINP compared to scintigraphy for the detection of the first BM, and additional investigations are needed. Nevertheless the published results indicate that PINP may be a useful marker for early detection and closer monitoring of skeletal involvement in prostate- and breast cancer patients. Even in BM of the lytic nature, as described above, highly increased levels of bone formation parameters have been identified. This emphasizes that more research into the coupling between bone resorption and bone formation may be important in detecting different types of BM.

**Coupling between osteoclasts and osteoblasts**

Interestingly, the coupling between bone resorption and bone formation was maintained in these prostate- and breast cancer patients +BM or –BM when analyzed separately with no differentiation between cancer types. Bone formation was significantly related to bone resorption and the number of osteoclasts; in the same manner bone resorption was significantly related to number of osteoclasts.

**ALPHA CTX-I as a resorption marker**

Levels of \(\alpha\alpha\)CTX-I may possibly originate mainly from bone metastases due to their high bone remodeling pathology, thus generating newly formed collagen type I matrix. Viewing the Spearman Rho coefficients it was noteworthy that in patients with BM, a higher correlation was observed between bone formation/number of osteoclasts \((r=0.84)\) and bone formation/bone
resorption \( (r= 0.84) \) than was observed for bone resorption/number of osteoclasts \( (r=0.76) \). This indicates that the number of osteoclasts and bone formation is more tightly coupled than the number of osteoclasts and bone resorption. Furthermore, the mean resorption activity on young bone per osteoclast was well correlated to bone formation in patients with BM \( (r=0.81) \) indicating that the resorption activity per osteoclast was not elevated in these patients if indeed bone formation is more tightly coupled to the number of osteoclasts rather than activity. However, further investigations are needed for such a conclusion and should be regarded as speculation. In patients without BM a similar picture was seen, however Spearman correlations were similar between all remodeling parameters, indicating that all were just as tightly coupled with regards to resorption of young bone, but to a smaller degree between resorption activity per osteoclast and bone formation.

**BETA CTX-I as a resorption marker**

Levels of \( \beta \text{CTX-I} \) may originate mainly from the total turnover of the skeleton due to the high bone turnover occurring locally in BM’s e.g. higher amount of newly formed bone. The Spearman Rho coefficients showed that \( \beta \text{CTX}, \text{PINP} \) and TRACP were just as strongly correlated in patients with and without BM. However, the mean resorption activity on aged bone per osteoclast was not correlated to bone formation in patients with bone metastases also indicating that bone formation is more tightly coupled to number of osteoclasts and not their activity in patients with bone metastases.

These data are somewhat in alignment with research suggesting that it is rather the presence of osteoclasts and not resorptive activity that is important for bone formation [240]. Compelling evidence for this is the fact that, in the normal adult skeleton, bone formation is almost exclusively initiated in areas having undergone resorption [85,86,230,231,256] indicating local signaling events between osteoclasts and osteoblasts. Consistent with this, the number of nuclei in osteoclasts has been shown to correlate to the number of osteoblasts [257]. Recently osteoclasts themselves were demonstrated to secrete bone anabolic signals[258]. This also led to speculation that resorbing osteoclasts do not always secrete the anabolic signal [259] implying that the anabolic signal is not derived exclusively during resorption of the bone matrix. Further strengthening this new view of the coupling between bone resorption and bone formation, was that bone resorption was less correlated to bone formation in cancer patients overall.

**Limitations**

An increasing number of publications have shown that biomarkers are potential candidates for a more dynamic evaluation of BM in breast- and prostate cancer patients [20,23,143,224-226] [2,22,25,27,227-229]. However, data derived from serum in our present study do not necessarily reflect the local environment at the BM site, but rather are mean values for total bone remodeling in the entire skeleton. They also do not necessarily reflect local coupling between bone formation and bone resorption, but throughout the body. In contrast, it may be that local events at the site
of metastases may reflect the systemic balance of bone resorption and bone formation, as cancer has been demonstrated to have an endocrine function [255,255,260] [261-263].

There are important differences in the pathology of osteolytic bone metastases (such as occur in breast cancer) and osteoblastic bone metastases [9,89,90,141], that most likely affect coupling between bone resorption and bone formation. Further investigations are needed to understand how the two different types of metastases affect coupling. The power of this study was not high enough to add to that discussion.

Another limitation is that this was a cross-sectional study and the statistical analysis was based on an assumption that the individual variation in biomarker levels in patients was low. Future analysis should be performed in a longitudinal study.

In conclusion, the present study provided further evidence that bone formation markers may be useful for obtaining valuable systemic information about the development of BM in breast- and prostate cancer patients. The bone formation marker PINP correlated well with the number of BM in these two types of cancer, but not in lung cancer patients. Furthermore, the study indicated coupling between bone resorption and bone formation is maintained in patients with breast or prostate cancer which has not been provided previously.
CHAPTER 6

A novel assay for assessment of extracellular matrix remodeling associated with liver fibrosis: An enzyme-linked immunosorbent assay (ELISA) detecting a MMP generated neo-epitope of type I collagen destroyed by cathepsin K cleavage

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To study collagen metabolism in liver fibrosis, and aid diagnosis and staging of liver fibrosis, a novel biomarker using competitive enzyme-linked immunosorbent assay (ELISA) has been developed to detect a collagen type I fragment generated by matrix metalloproteinases (MMP) -2, -9 and -13. Furthermore, we aimed at increasing the specificity of the test for non-skeletal collagen fragments by selecting an epitope, which was destroyed by cathepsin K cleavage.

Mass spectrometric analysis of collagen type I degraded by MMP-2, -3, -8, -9, -13 and Cat K revealed a large number of protease-generated neo-epitopes. From these data a fragment unique to collagen type I and generated by MMP-2, -9, -13, but destroyed by cleavage on the amino acid position 763 by Cat K, was selected as a target for ELISA development. Monoclonal antibodies were raised against this neo-epitope for assay development. The selected assay with the collagen type I neo-epitope cleaved at the amino acid position 763 (CO1-764) was evaluated in the preclinical rat model of liver fibrosis over four weeks: bile duct ligation (BDL) compared with sham-operated animals. The assay was further evaluated in a clinical study of prostate-, lung- and breast-cancer patients stratified according to skeletal metastases.

The CO1-764 marker was statistically elevated in BDL rats compared to baseline levels (p<0.05) and sham levels (p<0.01) at week 4 but not at week 2. We also found that the marker was destroyed by cathepsin K (Cat K) in vitro. Finally, the CO1-764 marker was not related to the skeletal involvement or number of bone metastases secondary to prostate, lung or breast cancer in a clinical study.

The ELISA detecting the collagen type I fragment, CO1-764, showed that this protein fragment generated by MMP-2, -9 and -13 may be destroyed by Cat K in vitro. This ELISA has the potential to describe the later stage of liver fibrosis.
Introduction

The extracellular matrix (ECM) is the most prominent component of connective tissue. It offers both the necessary structural support for cells to adhere and migrate to while its multifunctional proteins also play important signalling roles. The ECM mainly consists of collagens, proteoglycans and glycoproteins all of which play important and unique parts in maintaining the physicochemical structure of tissue [221,264]. ECM remodelling is a normal process in which formation and degradation of healthy tissue work synergistically in a balanced way. However in disease states an imbalance in the participating elements exists, which manifests itself in various pathologies. Excessive deposition of fibrillar ECM components, especially collagens, may occur in all tissues and organs, and is a significant cause of morbidity and mortality. During the development of fibrosis, the formation of ECM molecules occurs more rapidly than the degradation, leading to accumulation of fibrous tissue, i.e. scar tissue. Extensive tissue remodeling and fibrosis ultimately lead to organ failure resulting in death. This is most evident in end stage liver fibrosis, i.e. cirrhosis, a condition with high morbidity and mortality [265,266].

Fibrosis may begin in response to various acute or chronic stimuli, including infections, autoimmune reactions, toxins, radiation and mechanical injury [267]. The pathogenic process driving fibrogenesis is believed to be a dynamic series of events involving complex cellular and molecular mechanisms evolving from the acute or chronic activation of tissue repair activity that follows repeated tissue injury [221]. At present, no efficient and accurate markers of fibrosis diagnosis, staging and prognosis exist [268]. However, recently our group described a novel assay for the evaluation of liver fibrosis which involved assessment of a matrix metalloproteinase (MMP)-9 generated collagen type III fragment [269]. The N-terminal propetide of collagen type I (PINP) has also been shown to be elevated in fibrosis [267].

Endopeptidases such as MMPs play major roles in the degradation of extracellular macromolecules such as collagens and proteoglycans [270], resulting in protease-generated neo-epitopes. A neo-epitope is a protease-generated post-translational modification (PTM) [10, 11], and such neo-epitopes have potential as biochemical markers of ECM degradation. Assays developed with specific monoclononal antibodies to neo-epitopes, may aid the understanding of the temporal events leading to PTMs, and their potential role in liver fibrosis mechanisms. Neo-epitope-based biochemical markers found in urine and serum are receiving increasing attention for their diagnostic and prognostic potential [271]. This approach has been used in diseases such as osteoporosis, osteoarthritis, bone resorption and cartilage degradation markers [272]. However, neo-epitope-based biochemical markers describing liver fibrosis has not been studied extensively.

Fibril-forming collagens are synthesized as precursor molecules with large propeptide extensions at both the N- and C-termini of the molecule [273]. The mature propeptides are cleaved from
procollagen by N- or C-terminal proteinases, and mature collagen is integrated into the ECM [186,273]. During liver fibrosis there is an abnormal up-regulation in synthesis of collagen types I and III as well as MMPs [265]. Type I and III collagen levels increase by up to 8 times compared to their normal values [274]. With respect to excessive proteolytic activity in the fibrous tissue, the gelatinases MMP-2 and MMP-9 have been investigated and documented to be highly up-regulated [275,276]. Bile duct ligation (BDL) in rats has been used as a model of chronic liver injury due to its resemblance to hepatocyte damage, hepatic stellate cell (HSC) activation, and liver fibrosis observed in human cholestatic liver disease [265,277,278].

We set out with the hypothesis that MMP-2 and -9 mediated degradation of type I collagen may be of use for monitoring liver fibrosis. The scope of the present work was to develop a novel enzyme-linked immunosorbent assay (ELISA) for the assessment of MMP-2 and -9 degradation of collagen type I. By mass spectrometric analysis we selected a MMP-2 and -9 generated collagen type I neo-epitope that was destroyed by subsequent cathepsin K (Cat K) as a target. Since bone tissue mainly consists of collagen type I it would be well worth to eliminate the potential background coming from bone, which may be the case if the main protease of the bone resorbing cells osteoclasts cleaves collagen type I in the selected epitope (Figure 1). In theory, if the CO1-764 fragment is generated during bone resorption by MMP-9 produced by the osteoclast [279] it may lose antigenicity due to cleavage by Cat K, which is the main protease of osteoclasts [71].

**Figure 1.** Principle behind the double-neoepitope approach where an epitope is selected for A) its uniqueness for the target protein, B) the specific MMP generating it and C) the destruction of the epitope by Cat K which is relevant for bone matrix turnover.
MATERIALS AND METHODS

Reagents
All reagents used for experiments were standard high-quality chemicals from companies such as Merck (Whitehouse Station, NJ, USA) and Sigma Aldrich (St. Louis, MO, USA). The synthetic peptides used for monoclonal antibody production were purchased from the Chinese Peptide Company, Beijing, China.

In vitro cleavage
Purified collagen type I from human placenta (cat. no. ab7533, Abcam, Cambridge, UK) was cleaved with pro- MMP-2,-3,-8,-9 and -13 (cat. no. 444213-5; PF063-10; 444229-5; 444231-5; 444287-5; Calbiochem, Merck, Whitehouse Station, NJ, USA) and pro-Cat K (cat. no. 342001-10, Calbiochem, Merck, Whitehouse Station, NJ, USA). Pro-Cat K was activated using a solution of 50 µL of 100 mM NaAcetate/10 mM DL-dithiothreitol (DTT)/5 mM ethylenediaminetetraacetic acid (EDTA) pH 3.9. 50 µL of this solution was mixed with 50 µL pro-Cat K and incubated for 40 min at RT. 50 µg MMP was activated with 20 µL 1 mM 4-aminophenylmercuric Acetate (APMA) in dimethyl sulfoxide (DMSO) and incubated at 37°C for 2 hours. To facilitate MMP and Cat K cleavage of collagen type I, 1 mg/mL collagen type I diluted in 0.5 M acetic acid was dialyzed for two days to remove the acetic acid. The liquid was filtered to remove proteins below 10,000 kDa (Microcon Ultracel YM-10, cat. no. 42407, Millipore, Billerica, MA, USA). Each protease cleavage was performed separately by mixing 100 µg collagen type I and 1 µg of enzyme (MMP-2,-9,-13 or Cat K) in either MMP buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl_2, 2 mM Zn acetate, pH 8.0) or Cat K buffer (50 mM Na-Acetate, 20 mM L-cysteine, pH 5.5). As a control, 100 µg of collagen type I was mixed with MMP or Cat K buffer only. The collagen type I cleaved by Cat K was incubated for 24 hrs at 37°C. Each collagen type I aliquot cleaved by different MMPs was incubated for 3 days at 37°C. All cleavages were terminated using 1 µM cysteine protease inhibitor E64 (cat. no. 219377, Calbiochem, Merck, Whitehouse Station, NJ, USA). Finally the cleavage was verified by visualization using the SilverXpress® Silver Staining Kit (cat. no. LC6100, Invitrogen, Carlsbad, Ca, USA) according to the manufacturer’s instructions.
Peptide identification

Identification of peptide fragments in the in vitro cleaved samples was performed using matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography coupled to electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS). MALDI-TOF samples were purified using C_{18} zip-tips (cat.no.ZTC18SO24, Millipore, Billerica, MA, USA) according to specifications and 0.1 μg of material was eluted onto a MTP 384 ground steel target plate (Bruker-Daltonics, Bremen, Germany). MALDI tandem mass spectra were recorded on a Bruker ultraflex MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) in positive ion reflector mode. Mass spectra were externally calibrated in the m/z range of 800–4000 using peptides generated by tryptic digestion of bovine β-lactoglobulin. The m/z software “Flexanalysis” (Bruker-Daltonics, Bremen, Germany) was used to analyze spectra. LC-MS samples were ultra-filtrated to remove proteins above 10kDa, the pH was adjusted to 2.0 using formic acid, and 4 μL sample was analyzed by LC-MS/MS. LC was performed on a nanoACQUITY UPLC BEH C_{18} column (Waters, Milford, MA, USA) using a formic acid/acetonitril gradient. MS and MS/MS were performed on a Synapt High Definition Mass Spectrometry quadruple time of flight MS (QUAD-TOF; Waters, Milford, MA, USA), with an acquisition range of 350-1600 m/z in MS and 50-2000 m/z, in MS/MS. The software “ProteinLynx Global SERVER (PLGS)” (Waters, Milford, MA, USA) was used to analyze spectra and generate peak lists. To identify peptides, MS and MS/MS data was searched against a collagen type I (FASTA) protein database using the Mascot 2.2 (Matrix Science, Boston, MA, USA) software with either the MALDI-TOF/TOF or ESI-QUAD-TOF settings with carbamidomethyl (C), oxidation of methionine (M), oxidation of lysine (K) and oxidation of proline (P) as variable modifications.

Selection of peptide for immunizations

The first six amino acids of each free end of the sequences identified by MS were regarded as a neo-epitope generated by the protease in question. All protease-generated sequences were analyzed for homology and distance to other cleavage sites and then blasted for homology using the NPS@:network protein sequence analysis [280].

Immunization procedure

Six 4-6 week old Balb/C mice were immunized subcutaneously in the abdomen with 200 μL emulsified antigen 50 μg per immunization using Freund’s incomplete adjuvant (OVA-CGG-GSPGKDGVRG). Consecutive immunizations were performed at two week intervals, until stable titer levels were obtained. At each bleeding, the serum titer was investigated and the mouse with the highest titer was selected for fusion. The selected mice were boosted intravenously with 50 μg immunogen in 100 μL 0.9% sodium chloride solution three days before isolation of the spleen for cell fusion.
Fusion and antibody screening
The fusion procedure has been described elsewhere [281]. Briefly, mouse spleen cells were fused with SP2/0 myeloma fusion partner cells. The hybridoma cells were cloned using a semi-solid medium method and plated into 96-well microtiter plates for further growth. Here standard limited dilution was used to obtain monoclonal growth. Supernatants were screened using an indirect ELISA, while biotinylated peptide Biotin-K-GSPGKDGVVRG was used as a catcher peptide on streptavidin-coated microtitre plates.

Characterization of clones
Native reactivity and peptide binding of the monoclonal antibodies was evaluated by displacement of human serum, plasma and urine; rat serum and urine; and mouse serum, plasma and urine in a preliminary ELISA using 10 ng/mL biotinylated peptide coater on a streptavidin coated microtitre plate and the supernatant from the growing monoclonal hybridoma. The specificities of clones were tested towards free peptide (GSPGKDGVGRG), a non-sense peptide, and an elongated peptide (GSPGKDGVGRGL). Isotyping of the monoclonal antibodies was performed using the Clonotyping System-HRP kit, cat.5300-05 (Southern Biotech, Birmingham, AL, USA). Due to high homology, cross-reactivity toward a collagen type V sequence (GQKGDDGVRG) and collagen type VII sequence (TPVLDGVRG) was tested using synthesized peptides. The selected clones were purified using Protein G columns according to instructions (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK).

CO1-764 assay protocol
We labeled selected monoclonal antibodies with horseradish peroxidase (HRP) using the Lightning link HRP labeling kit according to the instructions of the manufacturer (Innovabioscience, Babraham, Cambridge, UK). A 96-well streptavidin plate was coated with biotinylated synthetic peptide Biotin-K-GSPGKDGVGRG dissolved in assay buffer (50 mM Tris, 1% BSA, 0.1% Tween-20, pH 7.4 adjusted at 4°C) and incubated 30 min at 20°C. 20 µL of peptide calibrator or sample were added to appropriate wells, followed by 100 µL of conjugated monoclonal antibody 4D3-HRP and incubated 1 hour at 20°C. Finally, 100 µL tetramethylbenzidine (TMB) (Kem-En-Tec cat.438OH) was added and the plate was incubated 15 minutes at 20 °C in the dark. All the above incubation steps included shaking at 300 rpm. After each incubation step the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The TMB reaction was stopped by adding 100 µL of stopping solution (1% HCl) and measured at 450 nm with 650 nm as the reference. A calibration curve was plotted using a 4-parametric mathematical fit model.

Technical evaluation
Linearity was determined by 2-fold dilutions of quality control (QC) serum, urine and plasma samples and linearity was calculated as a percentage of recovery of the 100% sample. The lower limit of detection (L DL) was determined from 21 zero samples (i.e. buffer) and calculated as the mean + 3x standard deviation. The inter- and intra-assay variation was determined by 10
independent runs of 5 QC samples, with each run consisting of two replicas of double determinations of the samples. Finally, for each assay a master calibrator prepared from synthetic peptides accurately quantified by amino acid analysis was used for calibration purposes.

**ELISA characterization**

The developed CO1-764 ELISA was evaluated using the materials described under “In vitro cleavage”. 20 µL from a sample diluted 1:20 was used for the assay. Cross reactivity was tested using the collagen type V and VII synthetic peptides using 20 µL 1000 ng/mL peptide solution for each test in the assay. Pre-cleaved MMP-9, which was subsequently cleaved by Cat K: 125 µL of the MMP-9 cleaved sample was mixed with 16.5 µL of activated Cat K (150 µg/mL in 100 mM NaAcetate, 10 mM DL-dithiothreitol (DTT), 5 mM EDTA, pH 3.9) and 237 µL activation buffer (50 mM Na-Acetate, 20 mM L-cystine, pH 4) to reach a final pH of 5.5. The mixture was incubated for 72 hrs at 37°C. The cleavage was terminated using 1 µM cysteine protease inhibitor E64 (cat.no.219377, Calbiochem, Merck, Whitehouse Station, NJ, USA). Finally the cleavage was verified by visualization using the SilverXpress® Silver Staining Kit (cat. no. LC6100, Invitrogen, Carlsbad, Ca, USA) according to the manufacturer’s instructions. The cleavages were repeated for three additional batches.

**Bile Duct Ligated Rat Model**

CO1-764 was assessed in a bile duct ligation rat model. The local ethical board had approved the study; approval #2008/561-1450. The study has been described in detail elsewhere [198]. Briefly, 17 female Sprague-Dawley rats aged 6 months were stratified into 4 groups. BDL- or sham-operated rats were sacrificed after 2 or 4 weeks. Liver fibrosis was induced in anaesthetized rats by standard BDL, in which the bile duct was ligated in two places and dissected between the two ligations prior to closing the abdomen. In sham-operated rats, the abdomen was closed without BDL surgery. Fasting blood samples were taken under CO₂/O₂ anaesthesia at baseline and at termination from the retro-orbital sinus of rats which had fasted for at least the previous 14 hours. The collected blood was left for 30 minutes at room temperature to clot, followed by centrifugation 2x at 1500 g for 10 minutes. The serum was then transferred to clean tubes and stored at -80°C until use.

The validity of the BDL model was demonstrated [25] by: 1) At the time of sacrifice livers of control animals showed normal gross morphology while livers of BDL animals were enlarged; 2) Mean liver weights were significantly increased in BDL rats compared to the sham-operated controls; 3) Semi-quantitative scoring of liver sections showed significantly more structural changes of the livers at weeks 2 and 4 compared to sham; 4) Histological examination of the livers of sham-operated animals showed that they were microscopically normal, with no sign of fibrosis whereas in BDL livers, a marked ductal proliferation was located around the portal tract and collagen deposition was found around the ductular structures. No other signs of cholestasis were seen,
whether intracellular cholestasis, bile plugs, bile infarction or hepatocytic rosette formation was observed; 5) by immunohistochemistry, collagen type I deposition was found exclusively in the venous wall of sham rats. In contrast, in week 4 BDL rats in which marked ductal proliferation was seen around the portal tract with the formation of multiple neo-bile ducts, more extensive type I collagen was found.

Cancer study design

CO1-764 was assessed in a cross-sectional study of patients with prostate, lung or breast cancer, with or without bone metastases (BM). The study design has been published previously [2]. Briefly, 90 breast-cancer patients (45 +BM and 45 -BM), 30 lung-cancer patients (16 +BM and 14 – BM) and 42 prostate cancer patients (25 +BM and 17 -BM) were referred to the Cancer Institute Hospital, Tokyo, Japan, between October 2002 and April 2004. All patients underwent bone scans using a radionuclide (Technetium-99m), as well as computer tomography (CT) and/or magnetic resonance imaging (MRI) to verify and quantify the presence of BMs. Both serum and urine samples were collected. All patients with skeletal complications were newly diagnosed and none had received therapies known to influence bone turnover in the 2 years prior to entry to the study. One breast cancer patient had also been diagnosed with Paget’s disease and was excluded from the analysis. All participants signed written consent forms and the study was performed in accordance with the Helsinki Declaration II and Standards of Good Clinical Practice. The Local Ethics Committee approved the study protocol.

Severity of metastatic bone disease (Soloway score)

The number of BM was recorded and the skeletal load was graded, as previously proposed by Soloway et al.[242]. Briefly, Soloway 0 refers to patients without BM, Soloway 1 to patients with fewer than 6 BM, Soloway 2 to patients with 6-20 BM, Soloway 3 to patients with more than 20 but less than a “super scan” defined involvement of more than 75% of the ribs, vertebrae, and pelvic bones; and Soloway 4 to patients with a “super scan”.

Statistical analyses

In the rat model the comparison of serum CO1-764 in BDL and sham rats at each time point was performed by analysis of variance (ANOVA) using the General Linear Models Procedure (GLM). For the cancer study the comparison of CO1-764 levels for each Soloway score against the level in patients without metastasis, the Dunnett’s adjustment of the level of significance was employed to correct for multiple comparisons. Differences were considered statistically significant if p<0.05. GRAPH PAD PRISM 5 (Graph Pad Software, La Jolla, CA, USA) was used for calculations.
RESULTS

*In vitro* cleavage and selection of peptides

All *in vitro* cleaved samples were visualized by silver staining to semi-quantitatively evaluate the degree of cleavage (Figure 2). High molecular bands of collagen type I were seen in the controls not exposed to enzymes, but these bands were severely degraded in the MMP-2, -9, -13 and Cat K cleaved samples, indicating that collagen type I was extensively degraded by these proteases. However, MMP-3 and MMP-8 samples retained the high molecular bands, indicating that these proteases were not able to degrade collagen type I extensively. Between 30 and 51 fragments of collagen type I cleaved by MMP-2, -9, -13 or Cat K, were identified with a statistically significant Mascot score (p<0.05). For collagen type I cleaved by MMP-3 and -8 the number of fragments identified with a statistically significant score was 5-7 (data not shown). All protease-generated neo-epitopes were tested for homology. Among many neo-epitopes the sequence 755’GSPGKDGV#R764 (CO1-764) in the alpha 1 chain of collagen type I generated by MMP-2, -9, -13 (Ụ) was found by LC-MS and selected for immunizations since it is unique to collagen type I and is further cleaved by Cat K (#) on the second position from the C-terminus of the peptide (GSPGKDGV#R). This sequence is 100% homologous to human, rat, mouse and bovine collagen type I.

![Figure 2. Silver staining of collagen type I cleaved separately by MMP-2, -3, -8, -9, -13, and Cat K.](image-url)
Clone characterization

The clone selected for ELISA development, was determined to be IgG2b subtype. The native reaction was high for this clone against both human serum and urine; rat serum and urine; and mouse serum (Figure 3). The signal was almost completely inhibited in all undiluted urine materials, and in human serum and plasma. For rodent serum and plasma the inhibition of the signal was around 70%.

![Standard curve](image1)

**Figure 3.** ELISA run showing typical standard curves and native reactivity against A) Human serum, plasma and urine, B) Rodent: Rat serum and urine; mouse serum, plasma and urine. Native material was run undiluted, 1:2, 1:4, 1:8 as indicated (---). The signal is seen as the optical density at 450 nm, subtracting the background at 650 nm, as a function of peptide concentration.

Technical evaluation

The typical standard curve is presented in Figure 3, showing a 4-parametric fit for the assay. The lower limit of detection (LDL) for the assay was 0.83 ng/mL. Dilution recovery was within 100±15% except for mouse urine (mean 118%) and mouse plasma (mean 125%) (Table 1). The inter- and intra-assay variation was around or below 10% for both assays (Table 2).
Table 1. Percentage dilution recovery for the CO1-764 assay. HS = human serum; HU = human urine; HP = human plasma; RS = rat serum; RU = rat urine; MS = mouse serum; MU = mouse urine; MP = mouse plasma.

<table>
<thead>
<tr>
<th>Serum CO1-764</th>
<th>HS</th>
<th>HU</th>
<th>HP</th>
<th>RS</th>
<th>RU</th>
<th>MS</th>
<th>MU</th>
<th>MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/mL</td>
<td>59.0</td>
<td>23.8</td>
<td>39.7</td>
<td>60.5</td>
<td>71.1</td>
<td>48.1</td>
<td>53.0</td>
<td>45.1</td>
</tr>
<tr>
<td>Undiluted</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
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<tr>
<td>Dilution 1:2</td>
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<td>100</td>
<td>99</td>
<td>100%</td>
<td>111</td>
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<tr>
<td>Dilution 1:4</td>
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<td>91</td>
<td>122</td>
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<tr>
<td>Dilution 1:8</td>
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<td>65</td>
<td>118</td>
<td>101</td>
<td>90</td>
<td>101</td>
<td>120</td>
<td>127</td>
</tr>
<tr>
<td>Mean</td>
<td>92</td>
<td>85</td>
<td>115</td>
<td>98</td>
<td>91</td>
<td>112</td>
<td>118</td>
<td>125</td>
</tr>
</tbody>
</table>

Table 2. Inter- and intra-assay variation for the CO1-764 assays using human serum and urine quality control samples. The variation was calculated as the mean variation between ten individual determinations of each sample.

<table>
<thead>
<tr>
<th>Serum CO1-764</th>
<th>Amount</th>
<th>Intra-assay variability %</th>
<th>Inter-assay variability %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>(ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HS1</td>
<td>19.7</td>
<td>11.1</td>
<td>5.3</td>
</tr>
<tr>
<td>HS2</td>
<td>22.2</td>
<td>11.8</td>
<td>7.5</td>
</tr>
<tr>
<td>HS3</td>
<td>22.9</td>
<td>11.4</td>
<td>6.7</td>
</tr>
<tr>
<td>HS4</td>
<td>24.4</td>
<td>10.1</td>
<td>6.8</td>
</tr>
<tr>
<td>HS5</td>
<td>28.4</td>
<td>10.3</td>
<td>5.5</td>
</tr>
<tr>
<td>HS6</td>
<td>29.2</td>
<td>9.7</td>
<td>6.2</td>
</tr>
<tr>
<td>HS7</td>
<td>38.0</td>
<td>8.7</td>
<td>6.8</td>
</tr>
<tr>
<td>HS8</td>
<td>54.9</td>
<td>6.8</td>
<td>8.4</td>
</tr>
<tr>
<td>Mean</td>
<td>10.1</td>
<td>6.7</td>
<td></td>
</tr>
</tbody>
</table>

ELISA characterization

From the ELISA characterization it was observed that MMP-2, -9 and -13 were able to generate the CO1-764 fragment (Figure 4). In contrast, MMP-3, -8 and Cat K were not able to generate this fragment. The fragment was not found in intact collagen type I. These findings were consistent in all three repeated cleaved batches. The test of whether Cat K destroys the CO1-764 fragment, showed that in a MMP-9 cleaved sample subsequently cleaved by Cat K, lost approximately 50% of the reactivity by subsequent cleavage of Cat K (Figure 4). Finally, no cross-reactivity was seen to the collagen type V and VII synthetic peptide sequences that have high homology with the immunization sequence of collagen type I.
**Figure 4.** Characterization of the CO1-764 assay with regards to reactivity against intact collagen type I (CO1 intact (1:20), collagen type I cleaved by MMP-2 (CO1/MMP-2 (1:20), collagen type I cleaved by MMP-3 (CO1/MMP-3 (1:20), collagen type I cleaved by MMP-8 (CO1/MMP-8 (1:20), collagen type I cleaved by MMP-9 (CO1/MMP-9 (1:20), collagen type I cleaved by MMP-13 (CO1/MMP-13 (1:20), collagen type I cleaved by cathepsin K (CO1/Cat K (1:20), collagen type I cleaved by MMP-9 corrected for dilution (CO1/MMP-9 1:40), collagen type I cleaved by MMP-9 and later Cathepsin K (CO1/MMP-9 + Cat K), elongated peptide and synthetic peptides corresponding to potential collagen type V (CO5) and collagen type VII (CO7) cross reactivity. 1:20 and 1:40 indicates the pre-dilution of the sample for assessment in the CO1-764 assay.

**Evaluation in the BDL study**

Levels of serum CO1-764 did not statistically change in the sham or BDL rats at the 2 week termination point however at the 4 week termination point it was seen that CO1-764 was significantly elevated in BDL rat compared to baseline levels (p<0.05) and compared to sham termination levels (p<0.01) (Figure 5).
Figure 5: Serum CO1-764 levels in the bile duct ligation (BDL) rat model for fibrosis. Serum CO1-764 was assessed in sham operated rats at baseline (sham-B) and termination (sham-T) as well as in BDL operated rats at baseline (BDL-B) and termination (BDL-T). Termination time points were 2 and 4 weeks after surgery. Asterisks indicate statistical significance as indicated by bars. (**=p<0.05; ***= p<0.001, ns = non-significant difference). Results shown are mean ± standard error of the mean (SEM).

Evaluation in the cancer study for bone metastases

Demographics of 161 cancer patients stratified according to cancer type and the presence or absence of BM have previously been reported [2]. There were no statistically significant differences in age and body mass index (BMI) between patients with or without BM. Figure 6A shows the mean values of CO1-764 in patients stratified according to cancer type and presence or absence of BM. There were no statistical differences in CO1-764 levels between patients without bone metastases and patients with bone metastases in any of the three cancer types.

Data from all cancer patients were pooled together for subsequent analyses using the Soloway scoring system for bone metastases, as previously reported. No linear associations had been found between Soloway score and the demographic characteristics of patients [2]. Figure 6B shows associations between Soloway score and CO1-764 levels. There was no relationship between advancing extent of the metastatic involvement of the skeletal system and CO1-764 levels.
Figure 6: CO1-764 levels in 161 breast-, lung- and prostate cancer patients stratified according to A) the type of cancer and +/- BM; B) the extent of metastatic bone disease described by the Soloway score 0 (-BM), and 1-4 (+BM). Results shown are mean ± standard error of the mean (SEM). Asterisks indicated highly significant elevated level in +BM patients compared to -BM, ns= non significant elevation in +BM patients as compared with −BM (**= p<0.001).

DISCUSSION

This is, to our knowledge, the first study to present the development of an assay detecting a MMP-2, -9 and -13 generated fragment, which is partly destroyed by Cat K thereby reducing the contribution of fragments from the skeletal compartment. Our main findings were: 1) The monoclonal antibody selected for assay development was highly specific towards CO1-764, a collagen type I fragment generated by MMP-2, -9 and -13, while the fragment was not generated by MMP-3, -8 or Cat K. The antibody did not cross-react with two highly homologous sequences of collagen type V and VII, indicating that the monoclonal antibody was specific for CO1-764; 2) The CO1-764 assay had a good native reactivity towards human serum, plasma and urine, rat serum and urine, and mouse serum, plasma and urine; 3) A technically robust assay was developed with acceptable inter-, and intra-assay variation, dilution recovery and a low limit of detection; 4) CO1-764 levels were significantly elevated in the BDL rat compared to baseline and sham levels at week 4 after surgery; 5) CO1-764 levels were not related to skeletal involvement in patients with prostate, lung or breast cancer.

Biochemical markers consisting of protein fragments from degrading tissue may indicate the disease pathology, which may be useful for diagnostic and prognostic purposes. Additionally, these markers may detect changes resulting from intervention strategies and serve as surrogate markers of efficacy [282]. Identification of biomarkers has become increasingly sophisticated during the last two decades due to continuous improvements in technologies for protein identification as well as expansion of proteomic information available. Large databases contain all known protein sequences, domains, size, post-translational modifications, and other information important for the understanding of protein function and which is imperative for selection of potential biomarker targets [164]. Software query tools enable comparisons of proteins for
determining uniqueness of sequences, alignments, species homology and so forth [165]. Collectively, all these databases provide key links for biomarker identification and target evaluations. Of the various techniques for biomarker analysis, mass spectrometry (MS) has become the most fundamental method for protein identification and thus biomarker discovery [166]. MS provides a high sensitivity, selectivity, and rapid analysis as well as interfacing with chromatographic methods, which has made it exceptionally well-suited for multi-analyte measurements in complex biological systems. This approach was applied in the present study to select an appropriate target for monoclonal antibody and assay development.

**In vitro cleavage and target selection**

The silver staining of the MMP and Cat K cleaved collagen type I revealed that the gelatinases MMP-2 and MMP-9, the collagenase-3 MMP-13 and the cysteine protease Cat K were able to efficiently degrade collagen type I in vitro, as expected from the literature [71,283,284]. In contrast, collagen type I was not extensively degraded by the stromelysin-1 MMP-3 and collagenase-2 MMP-8 in vitro. Others have found that MMP-8 as a whole enzyme was only able to act on a single specific site of collagen type I, which may be the case here, whereas the catalytic subunit alone was able to degrade at multiple sites [285]. MMP-3 is more active in degradation of the basement membrane and thus degradation of collagen type IV and laminin [286]. These data are all in agreement with MS data showing that the number of identified fragments was highest for the proteases having the highest activity towards collagen type I in vitro (data not shown).

**Selection of clones and ELISA development**

Characterization of the selected monoclonal antibody revealed strong reactivity towards human, mouse and rat fluids as well as with the CO1-764 peptide indicating that the antibody recognizes this amino acid sequence for collagen type I in native samples. It was seen that the native samples could be diluted to at least 1:8. Characterizations using the final ELISA format showed that the recognized peptide fragment indeed was generated by MMP-2, -9, and -13 but not MMP-3, -8 or Cat K. Furthermore, it was seen that the antibody was specific against the neo-epitope since no response was detected from intact collagen type I and the elongated peptide. These results were repeated for three different cleavage batches and were all in alignment with our previous MS findings regarding this cleavage site and which proteases generate these fragments. Even though the immunization sequence had a high homology toward a human collagen type V and type VII sequence, we did not detect any cross-reactivity to these sequences, further verifying the specificity toward the CO1-764 neo-epitope. Finally, Cat K was able to destroy the antigen in our in vitro study, which again was in complete alignment with our MS data. The 50% reduction in signal by Cat K may be attributed to technical issues since we were not able to switch to a Cat K buffer for the Cat K cleavage. Thus we diluted the MMP cleaved material using a Cat K recommended buffer ensuring that pH was correct, however the salts composition was exactly the same as recommended by the manufacturer.
Another MMP generated collagen type I marker has been evaluated for ECM related disease and liver fibrosis; the marker for the C-telopeptide of collagen type I (ICTP) [186,287-289], which was elevated in patient with cirrhosis [289]. However, this marker was developed using polyclonal antibodies and thus is not neo-epitope specific. Furthermore, it is generated by multiple MMPs and the specificity is not well established [186,194]. Technical evaluations of the competitive ELISA revealed that the assay was technically stable with a good dilution recovery and inter- and intra variation. However for mouse urine and plasma it was seen that the dilution was not as good as seen for other matrices. The highest sensitivity for the assay was observed between 5-50 ng/mL.

**CO1-764 for the evaluation of liver fibrosis**

In the BDL model of liver fibrosis, serum CO1-764 was elevated 4 weeks after BDL surgery compared to baseline and sham levels. This indicates that the neo-epitope assessed in this novel assay may be useful for the evaluation of liver fibrosis. These data are in agreement with the literature stating that especially collagen type I and II are highly elevated [198,268] and that MMP levels become elevated and unbalanced during fibrosis [265]. There are to our knowledge no reports in the literature on protease-generated neo-epitopes of collagen type I in fibrosis. Our data are however in agreement with previous findings showing that a MMP-9 generated fragment of collagen type III (serum CO3-610) is elevated in this BDL rat model, signifying that the ECM of the BDL liver is changed towards a more collagen-related profile. qPCR data in the same study also revealed that the synthesis of collagen type I is highly significantly elevated in the BDL livers compared to sham rats at both week 2 and 4 [198]. Nonetheless this fragment was also statistically elevated already at week 2 demonstrating that collagen type III and MMP-9 may become elevated when fibrosis is initiated [269]. These data indicate that the serum CO1-764 marker is not assessing general turnover but may be specific for liver turnover. The differences in the two assays CO3-610 and CO1-764 point towards that the two markers are not following the same pathologies.

**CO1-764 for the evaluation of bone metastases**

We investigated the ability of the CO1-764 marker to evaluate bone metastases secondary to primary prostate-, lung- or breast-cancer tumors. As in fibrosis, two of the key players impacting greatly on ECM remodeling in cancer proliferation are MMPs and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [96-98]. MMPs participate not only in the breakdown of ECM components and subsequent tissue remodeling, but also assist in regulation of the microenvironment [96-98]. Bone is the most common site of tumor metastasis in cancer [11]. The incidence of bone metastases is particularly common in breast and prostate cancer patients and arises when the primary tumor metastasizes to the bone causing a lesion where high bone remodeling occurs, consequently destroying the bone structure. MMP-2, -3, -9 and -13 are known to be elevated in the microenvironment of bone metastases secondary to different types of cancers such as breast and prostate cancer [111,125,290].
There were no statistical differences in urinary CO1-764 levels between patients with and without bone metastases in any of the three cancer types, clearly indicating no relationship between this marker and bone metastases. A similar picture was seen for CO1-764 in the same patients stratified according to the degree of skeletal involvement, using the Soloway score. It is well known that the vicious cycle occurring in bone metastases involves cancer cells prompting the bone cells — osteoclasts and osteoblasts — to become more active and thus a high bone turnover situation is reached [89,136]. The vicious cycle leads to a combination of collagen type I together with MMPs produced by the cancer cells and Cat K from osteoclasts from which CO1-764 may not be released due to the Cat K activity. This may explain why this marker is not related to bone metastases. The marker was also assessed in urine which may have an effect since the fragment assessed may be different. Several others markers have been reported by our group to be highly statistically related to the skeletal involvement in these patients [2], however this CO1-764 marker appeared not to be related to skeletal turnover.

In conclusion, we have developed an assay using a specific monoclonal antibody for the detection in human, rat and mouse fluids of CO1-764, a collagen type I fragment derived from MMP-2, -9 and -13 that was destroyed by Cat K in vitro. We demonstrated that CO1-764 was elevated in liver fibrosis but not in patients with prostate-, lung- and breast cancer and skeletal metastases. These data furthermore indicate that there is a high potential for the use of neo-epitope biomarkers for many ECM related diseases since this is well known from the arthritis and bone field [148], and has now been proven for liver fibrosis in the present paper and by Barascuk et al [269]. More studies are needed to further evaluate the potential of these markers to evaluate liver fibrosis.
CHAPTER 7

Is Bone Quality Associated with Collagen Age?

Collaboration between Leeming DJ¹, Henriksen K¹, Byrjalsen I¹, Qvist P¹, Madsen SH¹, Garnero P¹,²,³, Karsdal MA¹

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The World Health Organization defines osteoporosis as a systemic disease characterized by decreased bone tissue mass and microarchitectural deterioration, resulting in increased fracture risk. Since this statement, a significant amount of data has been generated showing that these two factors do not cover all risks for fracture. Other independent clinical factors, such as age, as well as aspects related to qualitative changes in bone tissue, are believed to play an important role. The term “bone quality” encompasses a variety of parameters, including the extent of mineralization, the number and distribution of microfractures, the extent of osteocyte apoptosis, and changes in collagen properties. The major mechanism controlling these qualitative factors is bone remodeling, which is tightly regulated by the osteoclast/osteoblast activity. We focus on the relationship between bone remodeling and changes in collagen properties, especially the extent of one post-translational modification. In vivo, measurements of the ratio between native and isomerized C-telopeptides of type I collagen provides an index of bone matrix age. Current preclinical and clinical studies suggests that this urinary ratio provides information about bone strength and fracture risk independent of bone mineral density and that it responds differently according to the type of therapy regulating bone turnover.
Introduction

In the description of osteoporosis presented in 1990 at the Consensus Development Conference, the disease was defined as a decrease in bone mineral density (BMD) and a microarchitectural deterioration of bone tissue leading to an increase in bone fragility and susceptibility to fracture [291]. Bone mineral density values at least 2.5 standard deviations below the mean levels in young adults are used to identify persons with osteoporosis [292]. Although numerous studies have shown that measurement of BMD alone is not sensitive enough to identify the majority of women who will sustain a fracture, the definition of osteoporosis has not changed [293-298]. Recently, a model combining BMD with clinical risk factors was proposed to improve the assessment of fracture probability [299]. This model, however, did not include factors related to bone remodeling and bone matrix quality.

Bone mineral density is currently the most important determinant of fracture risk [300,301]. Yet several studies have shown that up to 50% of persons who experience fracture have a BMD value above the level of 2.5 standard deviations below the reference-population mean, or a T-score > –2.5 [293,294,302]. Older persons can have up to a 10-fold increased 10-years fracture risk in comparison with younger individuals with the same BMD [303]: for example, an 80-year-old person with the same BMD as a 50-year-old has a 5-fold higher risk of hip fracture [303]. Thus, in individuals with comparable BMD, fracture risks are not the same. In addition, more than 50% of all incident fractures occur in women with osteopenia, as defined by a BMD T-score > –2.5 and < –1 [304]; at-risk women in this group will not be detected by applying the World Health Organization BMD definition of osteoporosis.

The bisphosphonate alendronate increases hip and spine BMD, leading to a lowering of the risk of vertebral fracture [295], never-the-less a meta-analysis showed that change in spine BMD accounted for only 16% of the vertebral fracture reduction [296]. Studies of the selective estrogen receptor modulator (SERM) raloxifene demonstrated that an increase in femoral neck BMD accounted for only 4% of vertebral fracture risk reduction [297]. Calcitonin treatment results in only a 1.2% increase in BMD of the lumbar spine, but it is associated with a significant 33% reduction in vertebral fractures [305]. Finally, changes in BMD associated with teriparatide (PTH) account for less than 40% of its effect in reducing vertebral fracture risk [306]. Together, these data demonstrate that changes in BMD with osteoporosis treatments only partially explain fracture risk reductions and that additional, independent factors contribute to the clinical efficacy of these therapies.

Bone quality has been extensively discussed in the literature [32,39,307-309]. However, the definition of bone quality remains imprecise, and in vivo assessment of its various components is nearly impossible to perform, as such assessment relies mainly on invasive techniques, which
consequently are not generally applicable in clinical trials. In this review we use the term “bone quality” as the quality of the bone matrix thus comprising mineralization, post-translational changes in the collagen molecule that occur with age and microscopic tissue damage which all are parameters affected by bone remodeling. Recent developments in the field of biochemical marker research have indicated that age-related changes in bone matrix molecules are associated with bone fracture resistance independent of BMD and may thus allow for the monitoring of some aspects of bone matrix quality in a manner that is non-invasive [29,32,39,202,310-315]. In this review, we emphasize the likely connection between bone matrix quality and bone remodeling, highlighting the importance of bone matrix age. Because determination of the age-related $\alpha$ to $\beta$ isomerization in the C-telopeptide of type I collagen ($\alpha\alpha/\beta\beta$CTX ratio) is currently the only method available for indexing mean bone age non-invasive [310,311,314], we discuss in detail the association between this index, bone matrix quality, and bone remodeling.

**What is bone matrix quality?**

Bone matrix is composed of mineral and organic matrix, mostly type I collagen. Bone matrix quality thus comprises the organization and extent of mineralization, structural changes in type I collagen molecules, and microscopic tissue damage occurring in this tissue. Central events regulating these properties of bone matrix quality are controlled by the rate of bone remodeling [176,309,316-318]. The mineralization of the bone matrix is dependent on the age of the bones, with older bones being more mineralized because of alterations in bone-remodeling rates [319]. The mode of action needed to increase the bone matrix strength is dependent on the existing level of mineralization of a given bone. If the mineralization is low initially an increase in mineralization would most likely benefit the bone strength in contrast to an already highly mineralized bone where an increase in mineralization most likely would decrease the strength of the bone [319-321]. The specific effects of changes in bone mineralization are unknown, although a study has indicated that microcracks preferentially occur in the highly mineralized areas of bones, i.e., the old bone [322]. Therefore, monitoring of the changes in bone mineralization, along with other parameters related to bone turnover, is essential to fully characterize bone matrix quality.

Also microcracks accumulate in bones with slow remodeling, i.e., when the age of the bones increases [323-325]. This accumulation is associated with loss of structural properties such as bone stiffness and energy absorption [326,327]. Although we are aware of the possible effect of number of microcracks and degree of mineralization on bone matrix quality further discussion of these properties is beyond the scope of this review in which we focus on the effect of post-translational changes on bone matrix quality.
a. Post-translational changes with age

Proteins in bone matrix, including type I collagen, undergo several nonenzymatic and enzymatic post-translational modifications with time. The rate at which these transformations occur and eventually accumulate in bone tissue is controlled by the level of bone remodeling [88,176]. This continuous remodeling of bone involves the function of osteoclasts, which act to achieve a coordinated and balanced resorption of aged bone, and of osteoblasts, responsible for sufficient formation of new bone, occurring in a localized, coordinated, and sequential manner referred to as coupling [240]. Secondary to inhibition of bone resorption, bone formation is decreased to a large degree by most anti-resorptives [240]. This leads to the generation of fewer new bone proteins, and as bone formation is inhibited, the age profile of the bone also increases. The result is accumulation of age-related post-translational modifications, such as isomerization ($\alpha\alpha/\beta\beta$ CTX ratio) and formation of advanced glycation end products (AGEs) and crosslinks. These post-translational modifications have been investigated in bone samples and systemically in urine samples, as they may be important determinants of bone strength and matrix quality [32,39,310,313,328,329]. Crosslinks, such as pyridinoline (PYD) and deoxypyridinoline (DPD), occur during the maturation of the bone collagen matrix (Figure 1C). AGEs are presented as protein-protein crosslinks (Figure 1D) and the concentration of the AGE pentosidine in trabecular bone of the vertebrae has been shown to contribute negatively to mechanical properties, and thus quality of the bone matrix [310],[330],[331],[332]. Because AGE, PYD and DPD are not as bone specific as the release of CTX, which is generated by cathepsin K [192], and that the accurate measurement of AGEs, in body fluid remains challenging, we focus on the urinary ratio of $\alpha\alpha/\beta\beta$CTX, which currently seems to be the most reliable marker to provide a noninvasive assessment of bone matrix quality.
Proteins containing an aspartate (D), asparagine (N), glutamate (E), or glutamine (Q) residue linked to a low-molecular-weight amino acid, such as glycine (G), can undergo a spontaneous nonenzymatic isomerization [154]. This isomerization introduces a kink in the conformation of the molecule, as the peptide backbone is redirected from the $\alpha$-carboxyl group in the native newly synthesized form to the side chain $\beta$-carboxyl [155]. The alpha 1 chain of type I collagen undergoes a $\beta$-isomerization in the DG motif within the CTX sequence ($^{1207}$EKAHDDGR$^{1214}$) of its C-terminal telopeptide (Figure 1A and B). This transformation has been shown to occur in vitro when bone matrix is incubated at 37°C and has also been documented in vivo in humans [155,202]. The CTX neoeptope is released during osteoclastic bone resorption by the action of cathepsin K (Figure 2A) and can be detected by an immunoassay. The native form $\alpha\alpha$CTX, composed of two crosslinked $\alpha$CTX peptides (one from each of the two alpha I chains of a collagen molecule), originates from newly synthesized collagen type I, whereas $\beta\beta$CTX is released from aged collagen type I. Thus, the ratio between $\alpha\alpha$- and $\beta\beta$-CTX gives an indication of the bone matrix age, maturation, and possibly quality (Figure 2B) [154]. As biochemical markers are measured systemically, the ratio between $\alpha\alpha$- and $\beta\beta$-CTX represents the sum of all sites where bone is being remodeled. It has
been shown that the mean age of bone assessed by $\alpha\alpha/\beta\beta$ CTX is lower in bone that is highly remodeled (trabecular bone) versus higher in less remodeled bone (cortical bone) [333]. *Ex vivo* experiments using bovine bone tissue or human vertebral bodies have also shown an association between the extent of type I collagen isomerization and mechanical properties, independent of BMD [313].

**Figure 2.** Schematic overview of the bone collagen age profile measured as the ratio between $\alpha\alpha$CTX and $\beta\beta$CTX. **A)** The osteoclast-specific Cat K–generated CTX epitope located in the C-terminal telopeptide of intact collagen type I exists in two isoforms: the $\alpha$- and $\beta$-forms. The endogenous age profile is reflected in the isomer composition. **B)** $\alpha\alpha$CTX and $\beta\beta$CTX fragments reflecting the endogenous bone collagen age are released from bone and can be assessed by ELISA. The ratio between $\alpha\alpha$CTX and $\beta\beta$CTX in urine samples reflects the mean bone collagen age, which may be regarded as an index of bone matrix quality. **C)** Theoretical bone age profiles in healthy individuals, patients with bone remodeling disorders, and patients during antiresorptive treatment.
b. Bone remodeling rates control aging of the bone matrix

To maintain optimal quality of bone tissue, continuous remodeling of the bone matrix is essential, and the rate of remodeling controls the age of the bone matrix [32,88,176,309,310,316-318]. The important contribution of bone remodeling as an independent determinant of fracture risk in both untreated and antiresorptive-treated subjects has been demonstrated using systemic biochemical markers [297,298,334,335]. Naturally occurring, rare genetic disorders of bone turnover also have revealed the effects of disrupted bone remodeling on fracture risk and bone matrix quality as changes in bone remodeling cause local alterations in bone tissue age (Figure 2C). Osteopetrosis and pycnodysostosis are characterized by absent or low osteoclastic bone resorption, high bone mass, and poor bone matrix quality, leading to increased fracture risk [236,336]. Conversely, in sclerotic diseases (e.g., sclerosteosis and Van Buchem disease), which result primarily from increased bone formation, the phenotype is associated with improved bone strength and lower fracture risk [337-339]. Similar findings have been reported in patients with autosomal dominant osteopetrosis type I; a high bone mass phenotype [340-342] associated with increased bone strength [342,343]. In Paget’s disease patients have a locally high remodeling rate with increased fracture risk thus impaired bone matrix quality [204]. These rare genetic disorders suggest that the expected association between high bone mass and improved bone strength is seen only when a certain level of osteoclastic bone resorption is maintained.

In summary, alteration of bone remodeling, particularly bone resorption, leads to pathological conditions characterized by alterations in bone matrix quality parameters, such as mineralization, and post-translational modifications all suggested to be associated with bone quality. As a consequence, increased fracture risk. This underlines the need for monitoring of bone remodeling and changes in bone matrix quality when assessing the effects of novel interventions on fracture risk.

Effects of osteoporotic treatments on bone matrix quality

Treatments for osteoporosis are targeted at reducing fracture risk by improving bone strength. Most available treatments target the resorptive capacity of the osteoclasts. Because bone formation is tightly coupled to bone resorption [240,256,344], most antiresorptive drugs decrease overall bone turnover [335]. In addition to eliminating the pathological excess of bone resorption, some antiresorptive treatments also alter targeted bone remodeling [240]. Targeted remodelling is the process ensuring that osteoclasts remove bone matrix where microcracks have occurred. The process involves osteocyte apoptosis in response to microdamage, which then in turn leads to activation of osteoclastic bone resorption, leading to removal of the damaged matrix, and finally new bone formation by the osteoblasts [29]. The outcome of such treatments would be an increase in the age of the bone matrix, eventually leading to accumulation of biochemical alterations in the collagen matrix as well as other aged related factors [202,310,311,329]. Such effects have raised major concerns within the field of bone research, especially with respect to the
massive suppression of bone remodeling, as seen with potent osteoporotic drugs [308,308,314,345]. Correlation of the change in overall bone turnover, assessed by the \( \alpha\alpha/\beta\beta \) CTX ratio, shows that bisphosphonates, which markedly reduce bone resorption and bone turnover, induce a decrease in the \( \alpha\alpha/\beta\beta \) CTX ratio, indicating an increase in bone tissue age (Figure 3). Drugs with more moderate effects on bone remodeling, such as SERM, HRT and calcitonin, do not or vaguely modify bone tissue age [311,314].

**Figure 3.** Change in \( \alpha\alpha/\beta\beta \) CTX ratio correlate to change in spinal BMD. BMD data have been adapted from phase III studies: PROOF study for calcitonin [305]; MORE study for SERM [346]; Women’s HOPE study for HRT [347,348]; FIT study for alendronate [193,349]; BONE study for ibandronate [201]. The \( \alpha\alpha/\beta\beta \) CTX data have been adapted from two other treatment studies [311,314].

The different antiresorptive therapies show evidence of varying impact on bone turnover and BMD, although their efficacy for the prevention of fracture, particularly vertebral fracture risk, is similar. SERM, HRT and calcitonin exhibit a 25%-50% reduction in bone turnover and 1%-3% BMD increase (Figure 4A and B), whereas potent bisphosphonates decrease bone resorption up to 70% of pretreatment levels leading to approximately 6%-7% increase in BMD [346-348,350-353]. Nevertheless, the reduction in fracture risk is similar among all these treatments [193,201,349,354,355], and SERM, HRT and calcitonin demonstrate antifracture efficacy approaching that obtained with the more potent antiresorptives [28]. Thus data indicate that changes in BMD during antiresorptive therapy do not directly translate to proportional decrease in fracture risk. We believe that changes in bone collagen maturation (\( \alpha\alpha/\beta\beta \) CTX), which is not captured by measurements of BMD, may also be involved. The bone matrix age decreases as bone turnover is decreased during anti resorptive treatments [311,314], (Figure 4C) indicating that bone matrix quality is compromised when using potent drugs such as bisphosphonates due to accumulation of aged bone matrix.
Figure 4. Effect of various antiresorptives on A) BMD<sub>spine</sub> increase and vertebral fracture risk reduction, B) systemic bone turnover (bone resorption) and vertebral fracture risk reduction, C) systemic $\alpha\alpha/\beta\beta$ CTX ratio and systemic bone turnover reduction (bone resorption). BMD, bone turnover, and fracture risk data have been adapted from phase III studies: PROOF study for calcitonin [305]; MORE study for SERM [209,346]; Women’s HOPE study for HRT [347,348]; FIT study for alendronate [193,349]; BONE study for ibandronate [201]. The $\alpha\alpha/\beta\beta$ CTX data have been adapted from two separate treatment studies [311,314].

a. Preclinical data

A recent study in skeletally mature female beagle dogs compared treatment with clinically relevant doses of alendronate, risendronate, raloxifene or vehicle for 1 year [328]. The bisphosphonates decreased the $\alpha\alpha/\beta\beta$ CTX ratio by 29%-56% in vertebral bone, compared with vehicle. In contrast, raloxifene did not change the age of the collagen matrix. Interestingly, the rate of bone turnover was significantly correlated with the concentration of the $\alpha\alpha/\beta\beta$ CTX ratio as well as other age related protein modifications with a higher rate of bone remodeling associated with lower bone collagen maturation. This finding indicates that administration of bisphosphonates leads to a more aged collagen profile in vertebral trabecular bone, compared with bone from SERM dogs which probably was due to the higher suppression of bone remodeling by the bisphosphonates. Other studies of the effect of SERM on bone matrix quality parameters confirm the absence of increased aging in both cortical and trabecular bone from femurs [356,357]. Several preclinical histomorphometric investigations have also proven that bisphosphonates reduce bone turnover to a much further extent than SERM and HRT [358,359]. In summary, preclinical data highlight the important finding that clinically relevant doses of drugs that markedly suppress bone remodeling lead to increased aging of the bone matrix.
b. Clinical data

A 2-year study of healthy postmenopausal women evaluated the effect of antiresorptive treatment on the systemic $\alpha\alpha/\beta\beta$CTX ratio as a measure of bone age [311]. Participants were treated with bisphosphonates, raloxifene, or HRT up to 24 months. The bisphosphonates alendronate and ibandronate induced a significantly higher bone age profile than for SERM and HRT (38%-52% vs. 3%-15% reduction in $\alpha\alpha/\beta\beta$CTX, respectively). These data indicate that the interventions have different effects on bone age, probably because of their different effects on bone remodeling. However, another study reported no change in the $\alpha\alpha/\beta\beta$CTX ratio in patients treated with alendronate for 2 years [360], possibly because of a lesser reduction in bone remodeling. In a similar manner the effect of oral calcitonin on the $\alpha\alpha/\beta\beta$ CTX ratio was assessed in postmenopausal women after 1 and 3 months of therapy [314]. Bone resorption was reduced by 30% but the $\alpha\alpha/\beta\beta$ CTX ratio was unchanged, suggesting that calcitonin does not affect the age profile of bone matrix. This is most likely due to the fact that calcitonin decreases bone resorption but either does not reduce bone formation, or shows a markedly lower suppression of bone formation than other anti-resorptive agents [361],[362],[363]. These data indicate that calcitonin does not eliminate the signaling between osteoclasts and osteoblasts which is referred to as “uncoupling” [364,365]. Thus bone formation is not largely decreased as seen with other drugs and the bone remodeling events remain.

The retrospective Risedronate and Alendronate (REAL) cohort study included women with osteoporosis who received either risedronate or alendronate [366] carried out for comparison of fracture risk reduction in the two groups. In the risedronate group, the risk of nonvertebral and vertebral fractures was 18% and 43% lower, respectively, than in the alendronate group. A trend was observed as early as 3 months after the initiation of therapy; at 6 months this difference was significant. Similar results were found in a meta-analysis of six trials of risedronate or alendronate therapy [367] in which the relative risk reduction for nonvertebral fractures was greater for risedronate than for alendronate. Although there are methodological limitations in the design of these analyses, the results suggest that risedronate may be more effective than alendronate in decreasing fracture risk, possibly because it reduces bone turnover to a lesser extent than alendronate and thereby preserves the bone quality properties of the matrix by a lesser increase in bone tissue age [193,349,368-370].

Concluding remarks

Bone remodeling is a key event in the maintenance of bone tissue mass, age, and quality. Assessment of the different components of bone matrix quality is currently hindered by the lack of specific, precise and importantly, non-invasive tests [311,314]. However, the preliminary data discussed here suggest that the use of the urinary $\alpha\alpha/\beta\beta$ CTX ratio can provide valuable information on the age, and thus the quality of bone matrix and on its changes during treatment. The relationship between changes in and absolute levels of the $\alpha\alpha/\beta\beta$ CTX ratio, on the one hand,
and the incidence of fracture in women receiving osteoporotic treatments, on the other, should be investigated, as associations may differ from those observed in untreated persons. Monitoring changes in the \( \alpha/\beta \) CTX ratio may then become a useful biological tool to assess the potential detrimental effects of some therapies, as there has been a concern that sustained long-term suppression of bone turnover may lead to increased bone fragility by compromising some aspects of bone matrix quality. It is interesting to note the renewed interest in drugs that modulate bone remodeling toward a more “steady-state” instead of leading to over suppression. It seems that a vast reduction in bone turnover in order to achieve gain in BMD compromises the quality of bone.

We propose that there is a relationship between the \( \alpha/\beta \) CTX ratio and the strength of the bone matrix; however additional data are still needed to demonstrate the causality of this relationship. Whether the \( \alpha/\beta \) CTX is a better marker than other urinary markers for evaluation of fracture risk reduction has yet to be evaluated in clinical studies with fractures as the primary endpoint. However, it has been demonstrated that the ratio between the two biochemical markers \( \alpha/\beta \)-CTX and \( \beta/\beta \)-CTX provides us with a different picture than observing one of the markers alone [311,314]. It provides a mean ratio of the bone matrix age that is resorbed in contrast to other bone remodeling markers that rather are index’s of the mean bone turnover, which will affect the bone matrix age.

Data discussed here indicate that BMD alone does not explain fracture risk but that we should rather consider the effect of increased BMD in combination with the bone matrix quality to obtain a more precise prediction of who will fracture (Figure 5A). The amount of bone mass that a patient has at a given time point in combination with knowledge about the age of the bone matrix could provide a better measure of fracture risk. We speculate that the optimal future treatments for bone loss require drugs that increase BMD while maintaining the rate of bone turnover thus bone age to preserve or increase the quality of bone during treatment as illustrated in figure 5B in contrast to current drugs that increase BMD at the expense of increasing the bone age due to decrease in the bone turnover. For the efficient development of novel osteoporosis drugs, monitoring of changes in BMD and bone remodeling as well as bone matrix quality should be considered, as these three factors are likely to provide important and complementary information on bone strength and fracture risk and to predict long-term safety.
Figure 5. Association between BMD, bone age (\(\alpha\alpha/\beta\beta\) CTX), bone matrix quality (Bone Q) and fracture risk (Fx). A) Impact of BMD and bone matrix quality on fracture risk. By considering the two effects, it may be possible to improve prediction of Fx (BMD × Bone Q = Fx). With current treatments it is speculated that when BMD goes up (+) the quality of bone goes down (-). B) Comparison of current and possible future treatments for bone turnover disorders and their effect on BMD, bone age and bone matrix quality. Current treatments increase BMD while lowering the bone age resulting in lowering bone matrix quality. Optimal future treatments should still increase BMD however without changing the bone age resulting in increase in bone matrix quality.
CHAPTER 8

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

*Citation*

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The aim of this review is to discuss the potential usefulness of a novel class of biochemical markers, designated neo-epitopes. Neo-epitopes are post-translational modifications (PTMs) of proteins and are formed by processes such as protease cleavage, citrullination, nitrosylation, glycosylation and isomerization. Each modification results from a specific local physiological or pathobiological 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 additional cancer-associated proteins and fragments are expressed. In addition, the severe cellular stress and inflammation caused by the cancer results in specific PTMs being distributed in the extra-cellular matrix (ECM).

We highlight the importance of remodeling of the ECM in cancer and the generation of PTMs that may be cancer-specific and reflect disease progression thus having potential for biochemical marker development.
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. This concept was proven by Mintz and colleagues, who showed that the normal mouse embryonic tissue micro-environment could repress expression of the tumor phenotype [371,372]. 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 activities and replaced by new, intact peptides and proteins. However, during pathological conditions, such as cancer, fibrosis and inflammation, this delicate repair-response balance is disturbed and tissue turnover becomes unsystematic [373,374]. 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 invasion the ECM may be stiffened and this can actually enhance tumor cell migration which has been observed using intra-vital imaging along type I collagen fibers adjacent to invading breast cancer cells [375,376].

During pathological remodeling of the ECM, excessive levels of tissue- and pathology-specific turnover products are released into the systemic circulation which, if detected in serum or urine, may be used as molecular biochemical markers of pathology. Turnover products holding these PTM modifications are released into the circulation during progression of cancer, and are referred to as neo-epitopes. Neo-epitopes 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 [146,377]. PTMs may be derived from processes such as aging (amino acid isomerization), citrullination, protease degradation and glycosylation [146,377]. Protease-generated neo-epitopes have, to date, received more attention than other PTMs. However, more interesting and potentially important PTMs that are relatively specific for cancer and other pathological conditions have recently been identified [146,378].

Recent studies suggest that PTMs may not only be useful biomarkers for early detection of malignant tumors, but they also contribute to abnormal cellular proliferation, adhesion characteristics and morphology [379] and may cause many of the differences between normal and cancer tissue [379-384] . Thus PTM profiles may be used as “biochemical footprints” for detecting and verifying the function and activity of key cellular signaling pathways [379-384].

The aim of this review is twofold. First, it highlights the importance of the ECM for controlling cell fate, and secondly 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 (3D) structure that surrounds cells [385], and consists of a network 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 consists primarily of collagen IV, laminins, entactin/nidogen and heparan sulfate proteoglycans [386]. 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 [387,388]. 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 [389].

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 trans-membrane signaling [372,390-393]. 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 [146]. 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 (3-dimensional appearance) of the ECM regulate the growth, remodeling, differentiation, migration and phenotype of a wide variety of cell and tissue types [394]. Although it is not clear how ECM topology and stiffness regulate cell fate, it is quickly becoming clear that these two parameters are likely to be as important as its biochemical composition in affecting cell behavior and influencing gene expression to regulate processes as disparate as stem cell differentiation and tumor phenotype.

ECM Remodeling

Remodeling of the ECM is essential for maintaining normal and healthy conditions of a given tissue. In the remodeling process in healthy individuals, old or damaged proteins are broken down by proteolytic activities and replaced by new, intact proteins. During pathological situations, such as cancer, fibrosis and inflammation, the balance is disturbed and tissue turnover becomes skewed, leading to an altered property of the ECM. Proteins having a major impact on ECM remodeling are the matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [96-98]. These proteins participate not only in the breakdown of ECM components and subsequent tissue remodeling, but also assist in regulation of the microenvironment [96-98]. MMPs fulfill these functions by modulating the availability and activity of growth factors and cytokines or their receptors, and by processing adhesion and signaling receptor targets [97]. In cancer, it has been found that overproduction of MMPs is associated with tumor initiation and progression in diverse tissues [97]. Although several mechanisms regulate
MMP expression, the final level of control is achieved by TIMPs. Intuitively, due to their inhibitory actions, members of this family should be able to inhibit cancer invasion and thus, be anti-tumor proteins.

The ECM controls cell phenotype
Tissue context influences malignant transformation and tumor progression [391]. This concept was demonstrated by Mintz and colleagues who showed that the normal mouse embryonic tissue microenvironment could repress expression of the tetracarcinoma tumor phenotype [371,372]. Bissell and colleagues then demonstrated that the normal chicken embryonic microenvironment could suppress transformation mediated by the Rous Sarcoma Virus (RSV) tumor oncogene, and that wounding promoted tumor progression [372]. Experimental data presented by multiple laboratories have since confirmed these observations and demonstrated that the tissue stroma can either promote or restrict tumor progression [98,395-397].

In humans, an altered ECM and hypoxia have been shown to contribute to tumor progression either directly, by destabilizing tissue integrity and promoting tumor cell motility, invasion and survival, or indirectly, by inducing tumor angiogenesis and enhancing tumor cell survival and selection [386,394,395]. This highlights the critical role of non-cellular microenvironmental factors in normal tissue homeostasis, and in tumor evolution and metastasis, and how the synergistic interaction between ECM remodeling and neo-epitopes of the non-cellular components of the tissue microenvironment, can cooperatively drive tumor metastasis.

In summary, while it is well understood that the phenotype results from the genotype, in some instances the opposite may apply. The phenotype may exert control over the genotype, suppressing malignant cancer cell proliferation and metastasis, if the matrix is intact.
**ECM in cancer –Related up-regulation 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 [398]. 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 [399]. 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 [399]. Matrix degradation can additionally promote malignant progression and metastasis.

ECM components and remodeling enzymes are elevated in the circulation in cancer patients [400,401]. Cancer is a disease caused by the disregard of essential rules governing how cells should organize in a stable manner within all living being tissue. 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 [374], 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 multi-organ failure and death. These processes affect the ECM as well as the proteases, cells and proteins found in the ECM. We will briefly review the most important ECM components affected during tumor growth and spreading of cancer cells.

**Proteases**

**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) [103]. 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 [107], resulting in extensive over-expression of a range of MMPs [108]. 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 [109]. MMPs also support angiogenesis and alter immune responses, blocking immune surveillance, with the overall effect of stimulating tumor growth [110]. An important controller of MMPs are the tissue inhibitors of the metalloproteinases (TIMPs). The TIMPs either directly inhibit the activity of MMPs by forming tight, non-covalent inhibitory complexes with them, or control the activation process itself [111]. In normal tissue turnover, MMP expression levels are at a low level apart from periods of rapid tissue remodeling, such as in
wound healing processes. 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, but also because 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 additional release of the urokinase form of plasminogen activator (uPA) by the endothelial cells of blood vessels surrounding the tumor, thus up-regulating both the proteolytic flow and angiogenesis [402].

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 [112]. 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 [113-123]. Irregular over-expression of MMPs has also been linked to metastasis of tumour cells in cancers including those of breast [124,125], colon [130] and lung [131]. It is noteworthy that there is not a single MMP that is consistently over-expressed in all tumor types, nor has a regular pattern of MMP-expression been seen among the variety of human cancers [122]. The expression of MMPs in cancer tumors mirror fundamental tissue heterogeneity, since various tumors express different subsets of ECM components, cell surface receptors, and cell tissue interactions. Nevertheless, has been reported that MMP-1, -2, -3, -7, -9, -11 and MT1-MMP (MMP-14) are frequently over-expressed in many human tumors [132].

**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 [99]. During normal conditions cathepsins are controlled by their endogenous inhibitors; the cystatins superfamily of protease inhibitors [100]. 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 [101]. In the literature there is evidence of their functional role in tumor growth, migration, invasion, angiogenesis and metastasis [102]. 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 [101,102].

**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 tenasin C. Collagen type IV, laminin, entactin, and certain proteoglycans are distinctively localized in the BM which divide organ parenchymal cells from the interstitial stroma [93]. 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 [94].

Collagen type I is the most abundant protein of the interstitial ECM, which is composed of two \( \alpha_1 \) chains and one \( \alpha_2 \) chain that pack together into thin fibrils. Cross-linking of collagen with other proteins is mediated via lysyl oxidase (LOX). The glycoproteins fibronectin and tenasin C modulate the integrin-mediated adhesion of cells to other ECM proteins, e.g. 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 [95]. The fibulins, Galectin-1 and Fibulin-1 function as intramolecular bridges in the organization of ECM supramolecular structures, such as elastic fibres and basement membranes [403]. Galectin-1 and Fibulin-1 can bind ECM components i.e. laminin, fibronectin, and therefore modify the adhesive properties of cancer cells [403-405]. 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 [406]. As a result, the integrity of the ECM is affected, and pro-inflammatory, pro-angiogenic and pro-metastatic factors from the ECM are released [407].

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 [288,408-412].

Matrix composition affects cell phenotype

The importance of matrix stiffness in tissue-specific differentiation is exemplified by the fact that cells grown as mono-layers (two dimensional: 2-D) 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 (3-D). Cells growing on plastic or glass are also unable to express differentiated proteins upon stimulation [394], or respond to growth factors or protease inhibitors in the same way as cells growing in a three dimensional setting [413]. These phenotypic disparities can be explained, in part, by the fact that living tissues in 3-D emit biological signals that may be read by specific integrins, but this signaling is non-existent in 2-D substrata such as tissue-culture plastic. Another illustration of this phenomenon is that when epithelial cells and melanocytes are grown in a 3-D ECM microenvironment, they assemble into tissue-like structures and express differentiated proteins when given the correct soluble stimuli [414]. Neither behavior is seen when the same cells are cultured on 2-D 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 [413]. For instance, osteoblasts grown on plastic in 2-D do not rely on MMPs for survival, whereas osteoblasts embedded in an interstitial matrix, such as 3-D type I collagen, are critically dependent for their survival on MMP-activation of latent TGF-beta [413]. 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 [415-417]. This 3-D contextual information is lost when cells are grown in 2-D.

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 [418-422]. Integrins are an excellent model on how an altered ECM could promote tumor progression. Integrins consist of 24 distinct trans-membrane heterodimers that relay cues from the surrounding ECM to regulate cell growth, survival, motility, invasion and differentiation [418-422]. 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 [423,424]. 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 [423,424].

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 a 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 non-coded, and unique parts of a molecule know as neo-epitopes [378]. Pathologically relevant protein modifications are not restricted to protease activity, although the subpopulation of neo-epitopes generated through this mechanism may be of paramount importance. Figure 1 depicts a handful of different types of PTMs. Some have been identified and used as biochemical markers as a measure of the disease activity [425], but also as contributions to disease process [378], as they change the functionality of the proteins.
Figure 1. 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.

Today, it is well-established that PTMs can uncover cryptic epitopes and/or create novel epitopes, that may initiate auto immune reactions [146]. Antigenicity and interactions of proteins with components of the immune system are possibly affected by PTMs, and modified self-antigens may be non-tolerated 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 [146,377,426-443]. These PTMs may be both early markers as well as pathological events leading to cancer and chronic inflammation [94,383,444,445]. 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 neo-epitopes 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 1A, 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 non-enzymatic pathways. Enzymatic cross-linking is often processed by the enzyme lysyl oxidase (LOX), which has been shown to promote the linearization of interstitial collagens, stiffing the tissues, and thus leads to neoplastic progression of tumor cells [149-152]. Interestingly, this matrix stiffness was associated with different phenotypes and enhanced mechano-responsiveness of the epithelium [149,151]. This highlights this PTM plays an important part in both the initiation and progression of metastasis.
Oxidations and hydroxylations

Oxidative damage to proteins is often caused by the action of 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 [153]. Oxidizing PTMs have been implicated in several pathological and healthy tissue turnover processes (figure 1B+C). 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 [446]. Under normal conditions these ROSs are strictly regulated by antioxidants, such as peroxidases and dimutases among others [447]. However, under pathological conditions oxidation may be implicated in tissue destruction. The role of ROS, in almost all aspects of cancer initiation and development [153,445,448-453] 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 neo-epitopes

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 1D). 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 [11;59;61;79;118;126]. Cartilage is for the most part composed of collagen type II, which accounts for 60%-70% of the dry weight of cartilage, and proteoglycans accounting for 10% of the dry weight, of which aggrecan is the most abundant [62]. 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 [106;119]. CTX-II is an MMP-generated neo-epitope derived from the C-terminal part of type II collagen [19;91], and measurement of CTX-II is highly useful for monitoring degradation of type II collagen in experimental set-ups assessing cartilage degradation [19;91;106]. Examples of other protease-generated collagen type II fragments selected as potential biomarkers for describing cartilage diseases are seen in figure 2. In addition, a range of protease-generated neo-epitopes 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 neo-
epitopes that have been developed and are used in both clinical and preclinical studies, were reviewed recently [106].

![Diagram of collagen structure](image)

**Figure 2.** Protease-generated neo-epitopes in collagen type 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.

Since many cancers are present in soft tissues of intestines and BM, identification of neo-epitopes 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 [288,412,454-456]. 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.

**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 non-enzymatic isomerization [154]. This isomerization introduces a kink in the conformation of the molecule, as the peptide backbone is redirected from the \(\alpha\)-carboxyl group in the native newly synthesized form to the side chain \(\beta\)-carboxyl [155] (Figure 1E). Peptides that contain amino acid isomerizations are often resistant to proteolysis [156,157] 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 [154]. In preclinical studies it has been shown that various known auto-antigens contain sites prone to deamidation and isomerization involved in type I diabetes, RA, systemic lupus erythematosus and experimental
autoimmune encephalomyelitis [157,457-460]. The C-telopeptide collagen type I marker CTX-I is a marker of bone resorption. It has been shown that assessment of the non-isomerized 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) [461]. 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 non-isomerized collagen type I is undergoing resorption by osteoclasts in this high turnover situation.

Non-enzymatic glycosylation

Non-enzymatic glycosylation is known as a Maillard reaction, and leads to post-translational modification of proteins, nucleic acids and lipids [462] (figure 1F). A common cause of non-enzymatic glycosylation is increased blood glucose levels, and accordingly most knowledge about non-enzymatic glycosylation arises from studies performed in diabetics [463]. 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 - i.e. nicotine - accumulation of AGEs is an inducer of cancer [464]. Furthermore, the receptor for AGEs, called RAGE, is currently under intense investigation as both a marker and an inducer of cancer [444], linking chronic inflammation and cancer [94,383,444,445,465].

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) [160,466]. The conversion of arginine into citrulline can have important consequences for the structure and function of proteins, since 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 [379]. 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 [467,468]. 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 histone deacetylase 1 (HDAC1) [467-469].

This highlights this class of PTMs, whether cellular or non-cellular, 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 neo-epitope 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 [2]. 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 3). All six remaining markers were significantly elevated in patients with Soloway score 1. The relative percent 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 [143]. 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 [3,145], 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) [145]. 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/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.
Biomarker platform for communication

Not all biochemical markers provide the same information. Some may have diagnostic utility, whereas others may indicate the potential efficacy of a therapeutic intervention. Thus, one biomarker that may fail when used for one purpose may provide important information in another application. An example is the Burden of Disease, Investigative, Prognostic, Efficacy of Intervention and Diagnostic (BIPED) classification, which provides specific biomarker definitions, with the goal of improving the development and analysis of OA biomarkers and of communicating advances within a common framework (figure 4). OA diagnostic biomarkers may be capable of identifying those in the general population with OA. Here a group at high risk of progressing may be identified. In this group of progressors, treatment efficacy could be monitored by an efficacy biomarker. A similar approach may be applied to the field of cancer diseases.

Figure 4. The three major categories of the BIPED classification system, which illustrate the types of uses of biochemical markers. Modified from Bauer et al., 2008.

Future directions

In this manuscript we have highlighted developments in protein chemistry, namely the combination of multiple disease-specific neo-epitopes that could be applied to clinical chemistry. The combination of multiple neo-epitopes 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 neo-epitope 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.

Figure 5. Schematic representation of the generation of neo-epitope 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, i.e. 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.
CHAPTER 9
Brief overview on additional data

I. Immunization programs initiated for collagen type I cleaved by MMPs and Cat K

Based on mass spectrometric analysis of peptides released from collagen type I upon protease digestion a number of cleavage sites could be established for MMPs and Cat K. The identified fragments with a significant MASCOT score are seen in Table 1-4. From these sequences, nine MMP neo-epitope where selected for antibody development (Table 5 and 6) according to homology to other collagens and whether an epitope was cleaved within the immunogenic sequence by other proteases in our analysis. All procedures for protein cleavage, target identification by mass spectrometry, peptide design, immunizations, monoclonal antibody productions, ELISA development and evaluations were as described in chapter 6.

Table 1. Identification of collagen type I fragments after MMP-2, -3, -8, -9, -13 and Cat K cleavage determined by MALDI-TOF/TOF (3 runs) using MASCOT for the database search. Variable modifications: Oxidation (M), Oxidation (K) = hydroxylysine, Oxidation (P) = hydroxyproline.

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Table 2. Identification of collagen type I fragments after MMP-2, -3 and -8 cleavage determined by LC-MS (ESI-QUAD-TOF) using MASCOT for the database search. Variable modifications: Oxidation (M) = hydroxylysine, Oxidation (P) = hydroxyproline.

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Table 4. Identification of collagen type I fragments after Cat K cleavage determined by LC-MS (ESI-QUAD-TOF) using MASCOT for the database search. Variable modifications: Oxidation (M), Oxidation (K) = hydroxylysine, Oxidation (P) = hydroxyproline.

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Table 5. Overview of peptides for use in immunizations and screenings in mice targeting collagen type I MMP cleaved neo-epitopes. Fragments noted here where identified by LC-MS during the present PhD thesis. Native seq. = A 10 amino acid peptide identified by MS including the protease generated site; Target seq. = six amino acids that are targeted for antibody production including a protease site; Immunogen= selected peptide coupled used for immunizations of mice; Screening seq.= peptide coupled to biotin used as a coater in ELISA for titer determination and screening of antibody producing hybridoma; De-selection seq.= peptides used for de-selecting antibodies with unwanted properties, here antibodies should not react towards peptides that have not been cleaved (elongated peptide); Characterization seq.= peptides used for characterization of screened antibodies.

<table>
<thead>
<tr>
<th>Collagen Type I</th>
<th>Species for target seq.</th>
<th>Native seq/ target seq.</th>
<th>Immunogen</th>
<th>Screening seq. (Selection)</th>
<th>De-selection seq.</th>
<th>Characterization Seq.</th>
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<td>CO1_MMP2/9/15</td>
<td>Human Target 100% to human, mouse, rat, bovine, canis c01</td>
<td>540 LTGSPGSPGP 490 / LTGSPG</td>
<td>LTGSPGSPGP-GGC-OVA (need to ensure some uncoated without BCC to use as standard curve)</td>
<td>LTGSPGSPGP-GGC-Biotin</td>
<td>GTGSPGSPGP (Biotin linked)</td>
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<tr>
<td>NB105</td>
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<tr>
<td>CO1_MMP2/9/13</td>
<td>Human Target 100% to human, mouse, rat, bovine, canis c01</td>
<td>271 5GLDGA4KGDADA 280 / 5GLDGA</td>
<td>5GLDGA4KGDADA - GGC-OVA (need to ensure some uncoated without BCC to use as standard curve)</td>
<td>5GLDGA4KGDADA - GGC-Biotin</td>
<td>FG5LDA4KGDADA (Biotin linked)</td>
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<td>NB104</td>
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<tr>
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<td>CO1_MMP3</td>
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<td>612 KDGEAGAQGP '621 / KDGEAG</td>
<td>KDGEAGAQGP - GGC-OVA (need to ensure some uncoated without BCC to use as standard curve)</td>
<td>KDGEAGAQGP - GGC-Biotin</td>
<td>KDGGEAGAQGP (Biotin linked)</td>
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<td>NB107</td>
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</tbody>
</table>

More check schemes
Only one Cat K site was in proximity of a MMP site and thus a possible ELISA sandwich partner to one of the MMP-generated neo-epitopes (Table 6). NB135 was designed to be a potential partner to the MMP-9 neo-epitope NB109 thereby combining an MMP site with a Cat K site to obtain specificity towards bone metastases; cancer cells producing MMP-9 and osteoclasts producing Cat K — both present in the vicious cycle. Unfortunately, no response was obtained for NB109 mice in two different rounds using first Balb/C mice and next CF-1 mouse strain. Thus it was not possible to develop such a sandwich ELISA and test this hypothesis.
**Table 6.** Overview of peptides for use in immunizations and screenings in mice targeting a collagen type I Cat K cleaved neo-epitope. The fragments noted here was identified by LC-MS during the present PhD thesis. Native seq. = A 10 amino acid peptide identified by MS including the protease generated site; Target seq. = six amino acids that are targeted for antibody production including a protease site; Immunogen= selected peptide coupled used for immunizations of mice; Screening seq.= peptide coupled to biotin used as a coater in ELISA for titer determination and screening of antibody producing hybridoma; De-selection seq.= peptides used for de-selecting antibodies with unwanted properties, here antibodies should not react towards peptides that have not been cleaved (elongated peptide); Characterization seq.= peptides used for characterization of screened antibodies.

**Cancer marker in combination with .QAGVMG for a sandwich partner to NB109**

<table>
<thead>
<tr>
<th>Collagen Type I</th>
<th>Species for target seq.</th>
<th>Native seq./target seq.</th>
<th>Immunogen</th>
<th>Screening seq. (Selection)</th>
<th>De-selection seq.</th>
<th>Characterization seq.</th>
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<tr>
<td>CO1_CatK NB135</td>
<td>Human Target 100% to human, mouse, rat, bovine, cat, rabbit CO1 + human CO4 Info: 0000GQGVMG (deamino mouse CO4)</td>
<td>601' PGPPGAVGPA, '610 /GAVGPA</td>
<td>OVA-CGG-PGPPGAVGPA</td>
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<td>PGPPGAVGPA</td>
<td>FGAVGPA (Human, bovine, mouse, rat CO4, mouse CO6)</td>
</tr>
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</table>

(please to order some uncoupled without GGG to use as standard curve)
II. Patent on protease generated neo-epitopes

A patent has been filed by Nordic Bioscience A/S for the protease generated neo-epitopes identified as described in the draft paper using LC-MS (chapter 6). All sequences described in section I of this chapter and other sequences from colleagues have been included. The patent has been filed as PJS/P16599WO at the European Patent Office and the US. Please see appendix 3 for more details. The patent is not official at present time.
III. Preliminary evaluation of the CO1-274 ELISA for the detection of bone metastases

The second assay that was developed within this Ph.d thesis was an assay against the NB104 sequence, where cleavage occurs at amino acid position 274 in the alpha 1 chain of collagen type I (CO1-274). The assay had not reached as far as the NB105 assay reported in the draft paper in chapter 6. However, a preliminary assay with good dilution recovery and variation was used for evaluating this markers relevance to bone metastases. The marker was evaluated in the prostate-, lung- and breast cancer study which also was used for the evaluation of the human PINP assay (Chapter 5) and evaluation of the CO1-764 (NB105) assay (Chapter 6). Please see a brief description of the study here and a more detailed description in Leeming et al.[2].

Materials and Methods Cancer study

CO1-274 using urine clone NB104-259 #3E5 was assessed in a cross-sectional prostate-, lung- and breast cancer study. The study design has been published previously [2]. Briefly, 90 breast cancer patients (45 +BM and 45 -BM), 30 lung cancer patients (16 +BM and 14 –BM) and 42 prostate cancer patients (25 +BM and 17 -BM) were referred to the Cancer Institute Hospital, Tokyo, Japan, between October 2002 and April 2004. All patients underwent bone scans using a radionuclide (Technetium-99m), as well as computer tomography (CT) and/or magnetic resonance imaging (MRI) to verify and quantify the presence of BMs. Both serum and urine samples were collected. All patients with skeletal complications were newly diagnosed and none had received therapies known to influence bone turnover in the previous 2 years prior to entry to the study. One breast cancer patient had also been diagnosed with Paget’s disease and was excluded from the analysis. All participants signed approved written consent and the study was performed in accordance with the Helsinki Declaration II and Standards of Good Clinical Practice. The Local Ethical Committee approved the study protocol.

Severity of metastatic bone disease (Soloway score): The number of BM was recorded and the skeletal load was graded, as previously proposed by Soloway et al.[242]. Briefly, Soloway 0 refers to patients without BM, Soloway 1 to patients with fewer than 6 BM, Soloway 2 to patients with 6-20 BM, Soloway 3 to patients with more than 20 but less than a “super scan” defined involvement of more than 75% of the ribs, vertebrae, and pelvic bones; and Soloway 4 to patients with a “super scan”.
Results Cancer study

From Figure 1 it was seen that this marker was not related to the presence of bone metastases or the number of bone metastases.

Figure 1: CO1-274 levels in 161 breast-, lung- and prostate cancer patients stratified according to A) the type of cancer and +/- BM; B) the extent of metastatic bone disease described by the Soloway score 0 (-BM), and 1-4 (+BM). Results shown are mean ± standard error of the mean (SEM).
CHAPTER 10

Concluding Remarks on the original papers 1-5.

**Figure 1.** Interactions between tumour cells, osteoblasts, and osteoclasts in the proximity of a bone metastasis. The resorption marker ALPHA C-telopeptide of collagen type I (CTX-I) and the bone formation marker N-terminal propeptide of collagen type I (PINP) are released during the vicious cycle. In contrast, the MMP-2,-9,-13 generated collagen type I neo-epitopes CO1-764 and CO1-274 where not related to bone metastases. Figure modified from Tanko et al [1].

1. (Paper 1) Urinary ALPHA CTX-I was shown to be specific for bone metastases revealing response to zoledronate treatment however not to docetaxel which is a treatment that targets the cancer cells thus all tumor sites also soft tissue tumors. (paper 1)

2. (Paper 2) A PINP serum assay for the human species and a PINP serum assay for the rat species using monoclonal antibodies were developed and it was verified that these indeed did monitor bone formation in both species. Both assays were also proven to be technical robust. (paper 2)
3. **(Paper 3)** The human PINP serum assay was evaluated in a study of prostate-, lung- and breast cancer patients with or without bone metastases determined by $^{99}\text{Tc}$ scintigraphy. The number of bone metastases was stratified according to the Soloway score e.g. number of bone metastases. It was found that PINP was elevated in prostate- and breast cancer patients with bone metastases compared to those without, however not in lung cancer patients. Furthermore, PINP was significantly correlated to number of bone metastases; highly statistical elevation was seen at all Soloway score 1-4, indicating that the first bone metastases could be detected. Nevertheless, it has not been shown that the marker is more sensitive than the imaging technique used in this case. (paper 3)

4. **(Paper 4)** A human/mouse/rat serum and urine assay (CO1-764) was developed for the assessment of a collagen type I fragment generated by MMP-2, -9, -13 cleaved at the amino acid position #764 in the alpha 1 chain. Antibodies were raised to be specific for the cleavage site. Furthermore, according to mass spectroscopy data, the epitope was cleaved by cathepsin K thus potentially destroying its antigenicity. The assay was proven technically robust. The CO1-764 assay was evaluated in a study of prostate-, lung- and breast cancer patients with or without bone metastases determined by $^{99}\text{Tc}$ scintigraphy. It was found that marker was not related to bone metastases; however it was associated to another extracellular matrix related disease - liver fibrosis. (paper 4)

5. **(Paper 5)** A preliminary human/mouse/rat serum assay (CO1-274) was developed for the assessment of a collagen type I fragment generated by MMP-2, -9, -13 cleaved at the amino acid position #274 in the alpha 1 chain. Antibodies were raised to be specific for the cleavage site. The CO1-274 assay was evaluated in a study of prostate-, lung- and breast cancer patients with or without bone metastases determined by $^{99}\text{Tc}$ scintigraphy. It was found that marker was not related to bone metastases. (discussed in chapter 9)
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An update on biomarkers of bone turnover and their utility in biomedical research and clinical practice

D. J. Leeming · P. Alexandersen · M. A. Karsdal · P. Qvist · S. Schaller · L. B. Tankó

Abstract Background: Maintenance of the structural and functional integrity of the skeleton is a critical function of a continuous remodeling driven by highly associated processes of bone resorption and synthetic activities driven by osteoclasts and osteoblasts, respectively. Acceleration of bone turnover, accompanied with a disruption of the coupling between these cellular activities, plays an established role in the pathogenesis of metabolic bone diseases, such as osteoporosis. During the past decades, major efforts have been dedicated to the development and clinical assessment of biochemical markers that can reflect the rate of bone turnover. Numerous studies have provided evidence that serum levels or urinary excretion of these biomarkers correlate with the rate of bone loss and fracture risk, proving them as useful tools for improving identification of high-risk patients.

Objective: The aim of the present review is to give an update on biomarkers of bone turnover and give an overview of their applications in epidemiological and clinical research.

Discussion: Special attention is given to their utility in clinical trials testing the efficacy of drugs for the treatment of osteoporosis and how they supplement bone mass measurements. Recent evidence suggests that biochemical markers may provide information on bone age that may have indirectly relates to bone quality; the latter is receiving increasing attention. A more targeted use of biomarkers could further optimize identification of high-risk patients, the process of drug discovery, and monitoring of the efficacy of osteoporosis treatment in clinical settings.

Keywords Bone resorption markers · Bone formation markers · Bone regulatory proteins · Bone mineral density · Fracture risk · Treatment · Monitoring · Postmenopausal women

Introduction

Osteoporosis and the related fragility fractures remain major epidemiological burdens of postmenopausal women. Currently, the prevalence of osteoporosis in industrialized countries is estimated to be 40% in women in their sixties, and 70% in women in their eighties [25]. Elderly men also lose bone with aging and, due to increasing longevity of the elderly, more and more men reach the state of osteoporosis and have increased risk of fragility fractures. The estimated lifetime risk of fractures in men over 50 years of age is estimated to be 17% [78]. The impact of osteoporotic fractures on morbidity and mortality is greater in elderly men than in women [3], emphasizing the need to give a bi-gender focused consideration to the clinical management of osteoporosis.

A central component of the pathogenesis of osteoporosis is an imbalance between the function of two key players of bone turnover, namely osteoclasts and osteoblasts. Estrogen deficiency arising after the menopause leads to acceleration of bone turnover, the rate of bone resorption exceeding the rate of bone formation. This leads to a net negative calcium balance and consequent demineralization of bone [54].

The rate of bone resorption and formation can be estimated by immunoassays measuring the serum concentration or urinary excretion of different target molecules specific to these cellular processes [27]. Over the past decade, a wide array of such immunoassays has been launched for in vitro, ex vivo, and in vivo investigations. Their systematic validation led to the recognition of their utility for assisting biomedical research, targeting the better understanding of the pathogenesis of osteoporosis, the improvement of clinical diagnostic, and the evaluation of novel treatment modalities.
The aim of this review is to provide an up-to-date summary of recent developments in the field of bone markers and to discuss their utility in assisting prediction of fracture, drug development, and monitoring of treatment efficacy.

Cellular and structural elements of bone

Bone is composed of type I collagen fibres, crystals of hydroxyapatite \([3Ca_3(PO_4)_2]·(OH)_2\], and ground substance. Based on structural differences, bone can be subdivided into cortical and trabecular compartments. Cortical bone (the outer layer) is composed of a thick and dense layer of calcified tissue, whereas trabecular bone (the central part) is composed of thin trabeculae forming a robust, though slightly flexible, framework \([5]\). Bone homeostasis is critical for maintenance of bone strength and endurance. During the remodeling process of bone, osteoclasts and osteoblasts interconnect in the Basic Multicellular Units (BMU), to degrade old bone and replace it with the exact same amount of new bone. In premenopausal women, the activity of osteoblasts and osteoclasts are balanced so that the net result parallel resorption and formation is zero \([63]\). However, after the menopause, coupling between bone resorption and formation becomes partially disrupted, i.e. the rate of resorption increases more pronouncedly than the rate of bone formation, which in turn leads to a negative calcium balance and consequent bone loss. Continuous bone loss leads to low bone mass and micro-architectural deterioration of bone tissue that enhances bone fragility and leads to an increased risk of fractures \([24]\).

Biochemical markers of bone turnover

Markers of bone turnover can be stratified regarding their origination from the BMU Fig. 2:

a. **Collagenous bone resorption markers**: Measures of collagen type I degradation products released during osteoclastic resorption of bone.

b. **Osteoclast regulatory proteins markers**: Proteins that either regulates the differentiation and proliferation of osteoclast precursors into mature osteoclasts or are involved in the coupling between osteoblasts and osteoclasts.

c. **Bone formation markers**: Measures of enzymatic activity of osteoblasts, bone proteins and fragments of pro-collagens released during bone formation.

Collagenous bone resorption markers

The most common markers of bone resorption measure peptide fragments deriving from collagen type I, such as CTX-I, NTx, ICTP, and pyridinolines. Collagenous markers reflect the rate of bone resorption, but may also provide information on the composition and thereby quality of bone \([12]\). The location of the collagen derived resorption fragments are denoted in Fig. 1.

**CTX** is an 8-amino acid fragment from the C-telopeptide of type I collagen (Fig. 1). CTX is generated by cathepsin K activity and the rate of its release from bone is a useful reflection of the resorbing activity of osteoclasts \([9]\). 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; the lower the ratio, the older the bone tissue \([36]\). Resorption rate of newly synthesized collagen type I can be assessed by specific immunoassays targeting the detection of αCTX in urine samples \([23]\). Degradation rate of matured, isomerized, collagen can be estimated by another specific assay targeting βCTX in both urine and serum samples. The intra- and inter-assay variations (CV%) for these assays are <9% \([23, 88]\).

**NTx** is an 8 amino acid epitope (JYDGKGVG; Fig. 1) derived from the N-telopeptide of type I collagen \([48]\). This fragment is cleaved by cathepsin K and the rate of its release is also a useful measure of bone resorption. NTx can be measured in serum and urine by a specific immunoassay. The intra- and inter-assay variations are 6.1% and 4%, respectively \([48]\).

**ICTP** measures a relatively large hydrophobic phenylalanine-rich pyridinolines cross-link of the two α-1 chain in the C-terminal telopeptides of matured collagen type I \([86]\). The ICTP epitope neighbours CTX (Fig. 1) but it is released as a result of MMP activity \([42]\). Cathepsin K activity eradicates the ICTP epitope. ICTP can be measured in serum and plasma. The inter- and intra-assay variations of the assay were reported to be between 3 and 8% \([86]\).

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Fig. 1 Structure of type I pro-collagen and mature type I collagen. Location of the collagen epitopes are indicated by arrows.
PYR (deoxypyridinoline and pyridinolines) are found in mature type I collagen and involved in the formation of cross-links between adjacent collagen polypeptides. Pyridinium cross-links do not appear to be metabolised. Because these cross-links are formed during the late stage of fibril formation, measurements of these are considered as an index for degradation of mature collagen [38]. Pyridinolines may be detected in both serum and urine by specific immunoassays. The intra- and inter-assay variation for one DPD assay has been reported to be 4–8% and 3–5%, respectively [69].

Osteoclast regulatory proteins

The transition of osteoclast precursors to mature osteoclasts that are capable of resorbing bone is tightly regulated by osteoclast regulatory proteins Fig. 2. The OPG/RANKL/ RANK cytokine system is essential for osteoclast biology. A number of studies point out that alteration of this system is involved in the pathogenesis of metabolic bone diseases. Estrogen deficiency is accompanied by increases in RANKL and decreases in OPG levels leading to increased number and lifespan of mature osteoclasts [52]. Accordingly, resorption will increase and bone loss becomes evident with time. Consequently, the OPG to RANKL ratio is of great interest and may be a more powerful indicator of osteoclast activity than separate evaluation of the two proteins.

Markers of osteoclastogenesis are relatively new players that are not fully characterized regarding their potentials in biomedical research and clinical trials of osteoporosis treatment. However, preliminary observations suggest that they can be helpful in the elucidation of the mechanism of action and efficacy of novel drugs acting on osteoclast function. Markers of osteoclastogenesis include RANKL and OPG, whereas markers of osteoclast number include TRAcP and Cat K.

**Markers of osteoclastogenesis**

RANKL (receptor activator of nuclear factor NF-κB ligand) is a member of the tumor necrosis factor (TNF) family and is produced by bone-forming osteoblasts and activated T lymphocytes. It activates its specific receptor RANK on osteoclasts precursors, thereby promoting cellular maturation in the presence of macrophage colony-stimulation factor (MCS-F). It is the main mediator of osteoclast activation, differentiation, and survival [59, 62, 100]. RANKL expression is inversely correlated with serum levels of 17β-estradiol and positively correlated with bone resorption markers [52] and may therefore be considered as a measure of osteoclast activity. As an example the intra- and inter-assay variation for one total RANKL kit (Immundiagnostik, Bensheim, DE) is 2.2% and 8.2%, respectively.

Osteoprotegerin (OPG) is a soluble decoy-receptor, which is produced in different tissues, e.g., bone, liver, stomach, intestine, and lung. Osteoblasts secrete OPG that binds to RANKL and thereby inhibit the regulatory effect of RANKL on osteoclast activation and proliferation [51, 99]. OPG production is positively correlated with estrogen levels [60]. Intra- and inter-assay variation for an available OPG assay (Immundiagnostik, Bensheim, Germany) is <10%.

**Fig. 2** Schematic presentation of type I collagenous, pro-collagenous non-collagenous and osteoclast regulatory proteins released during the activity of a BMU. Markers released during osteoclastogenesis (red), bone resorption (blue) and bone formation (black) are indicated by arrows.
Markers of osteoclast number

*TRA*P (tartrate-resistant acid phosphatase) is a glycoprotein produced in mature osteoclasts, activated macrophages and dendritic cells. It is active as a phosphatase and as a generator of reactive oxygen species [76]. The polypeptide chain of TRAcP is cleaved by proteases into two isoforms 5a and 5b, which activate phosphatase activity [76]. The isoform TRAcP 5b is derived from osteoclasts and has been proposed to reflect osteoclast number rather than bone resorption [2, 17]. It is known that TRAcP 5b increases with age in healthy women and after the menopause [15], hence TRAcP 5b may be useful in clinical trials evaluating novel treatments of osteoporosis. TRAcP 5b can be measured in serum samples. The intra- and inter-assay variation of two selected TRAcP 5b assays were 2.1–7.9% and 4.9–13%, respectively [74].

Cathepsin K is a member of the cysteine protease family. This enzyme plays a critical role in osteoclastic degradation of collagen type I. Dissolution of the inorganic phase of bone in the resorption lacunae at low pH is a prerequisite for the degradation of the organic phase, which is mainly mediated by cathepsin K [57]. The enzyme is secreted into the lacunae, where it cleaves both helicoidal and telopeptide regions of the collagen molecules. Cathepsin K is an abundantly synthesized by mature resorbing osteoclasts [53]. This marker can be measured in serum samples. The intra-and inter-assay variation range from 4% to 8% [53].

Bone formation markers

Formation of bone is most often evaluated using the following biomarkers: bone specific alkaline phosphatase, osteocalcin, or PICP/PINP.

Enzyme activity markers

**Bone specific alkaline phosphatase (BSAP)** Approximately half of serum alkaline phosphatase activity comes from the bone-specific isoenzyme [89]. BSAP concentrations demonstrate a linear relationship with osteoblast and osteoblastic precursor activity. During the immediate post-proliferative period (12–18 days), the bone extracellular matrix endures a succession of modifications rendering competence for mineralization. In cultures that progress into the mineralization stage, all cells become alkaline phosphatase (AP) positive immunohistochemically, indicating that AP is involved in the mineralization of bone. Serum concentration of BSAP may be assessed indirectly after precipitation with lectin. The intra-assay and inter-assay CVs are <4% and <10%, respectively [89].

**Bone protein markers**

Osteocalcin (OC, former Gla-protein) is synthesized and secreted by osteoblasts and constitutes the major non-collagenous protein of bone matrix [102]. The physiological role of osteocalcin is related to its high affinity to calcium, thereby changing the osteocalcin polypeptide (a 49 amino-acid residues) into a compact α-helical confirmation in which the glutamic acid residues stimulate the absorption to hydroxyapatite within bone matrix [102]. In both preclinical and clinical studies, circulating OC has been shown to correlate with histomorphometric measurements [33, 55, 91]. Using specific immunoassays, osteocalcin can be quantified in plasma or serum samples. Intra- and inter assay for osteocalcin assays have been reported to be 5.7–6.4% and 5.9–6.1% [88], <8% and <15% [72], <2.3% and 2.5% [79], respectively.

Pro-collagen markers

PICP/PINP represent the C- and N-terminal pro-peptides of type I collagen, respectively. These pro-peptides are trimeric, globular, peptides that are enzymatically released from newly synthesized pro-collagen prior to its incorporation into the extracellular matrix. Circulating levels of PINP have also been demonstrated to correlate directly with histomorphometric indices of bone formation [35]. PICP and PINP can be detected in serum/plasma by specific assays. The intra- and inter-assay variation for a PINP ELISA was 4.6–5.3% and 2.9–4.9% [77], respectively. For a PICP ELISA, these parameters were 5–7% and 5–7% [69].

Variation of biochemical markers

Collagenous markers of bone resorption have been reported to exhibit marked biological circadian variation with a nadir during the day and in the afternoon with a peak during the night and early morning hours [80, 94]. Thus, the increased bone resorption occurring at night is counterbalanced by an equally large inhibition of bone resorption during the day (the ‘area-under-the-curve’ being zero over a 24-h period in premenopausal women). Circadian variation is independent of gender, age, menopausal status (although the baseline level of bone degradation in postmenopausal women is higher than before the menopause), mobility, vision, and pituitary hormone secretion [95]. Recently, the major component of the circadian variation in resorption markers has been attributed to the effects of food intake, probably involving endogenous secretion of glucagon-like peptide 2 (GLP-2) [50]. The cause of circadian variation remains unknown, but it is speculated that serum calcium homeostasis is crucial for the nature of this phenomenon [50]. Consequently, in order to obtain valid estimates of bone resorption markers, blood and urine sampling must be performed in the fasting state of individuals and within relatively narrow time frames (0800–1000 hours). Circadian variability of approximately 10–20% has been found for serum OC and serum BSAP, and approximately 40% for the urinary resorption marker NTx and 60–66% for serum CTX [21].
The use of biochemical markers for risk prediction

In this section we will discuss biochemical markers in relation to:

a. BMD and bone loss
b. Fracture risk

BMD and bone loss

With the exception of a few [6, 58], most clinical investigations support the existence of an inverse correlation between bone turnover markers and BMD. Several prospective studies have shown significant correlation between levels of bone turnover markers and rates of bone loss assessed by serial BMD or BMC measurements at different skeletal sites over 1–13 years [98], indicating that bone turnover markers may provide additional information to BMD measurements. Whereas, in premenopausal women, bone turnover rates account only for 0–10% of the variation in bone mass, this percentage increases up to 52% in elderly women [38]. After the menopause, the inverse association of BMD with biochemical markers of bone turnover becomes stronger with advancing age [28], and stronger for resorption markers than for formation markers. In groups of untreated postmenopausal women, investigators found a significant correlation between baseline measures of bone turnover markers and the subsequent rate of bone loss at the hip or wrist [6, 68], but apparently not at the lumbar spine [98].

Explanations of this latter finding rests in methodological limitations of DEXA scanning in the elderly. After the age of 65 years, presence of progressive vascular calcification in the lumbar aorta and degenerative changes of the lumbar spine interfere with objective measurement of BMD. Monitoring over time reveals increases rather than decreases as is otherwise seen at peripheral skeletal sites [97]. A prospective study has shown that increased levels of biochemical markers could identify a subgroup of subjects who were ‘rapid bone losers’ (i.e. >3% loss in BMD per year) in the subsequent 2–12 years [4]. However, biochemical markers of bone turnover alone are not suitable for estimating BMD, bone loss, or fracture risk in an individual subject, although they might be useful as supplements to BMD measurements. The conflicting opinions regarding the ability of bone turnover markers to predict bone loss is mainly focused on the lumbar spine and to some extent prediction of hip BMD in individuals [6] and the predictive value of a single measurement of these markers [58]. We believe that bone turnover markers are not optimal in predicting bone loss in elderly patients, due to the above-mentioned reasons in the aorta, and that serial measurements of markers should be performed in order to predict bone loss.

In a 4-year prospective study of 305 women from the OFELY cohort (mean age 64 years), bone resorption (NTx, CTX) and formation markers (OC, PINP) were evaluated [40]. Baseline values of bone turnover markers were highly correlated with the rate of BMD loss in the forearm ($r = -0.19$ to $-0.30$, $p<0.001$), independent of age. In early postmenopausal women (years since menopause≤5 years) with the highest rate of bone loss, the correlation coefficient increased to 0.53. Another prospective study examined the ability of formation markers (OC, BSAP) and resorption markers (NTx, PYR, D-PYR CTX) to predict hip bone loss in 295 elderly women (age ≥67 years) [6]. Increased levels of all four resorption markers were significantly associated with fast rates of bone loss at the total hip, although not at the femoral neck. Women with OC levels above the median value were also associated with increased rate of bone loss, whereas BSAP did not seem to provide information on the rate of hip bone loss. In a recent 5-year follow-up study, including 429 pre-and postmenopausal women by Lofman et al., it was found that formation markers (OC, ALP) and resorption markers (hydroxyproline, calcium) at baseline correlated significantly with BMD at 5 years at group level [65] indicating that biochemical markers of bone turnover provides information about future bone loss.

In a study of 105 male individuals, 65 osteoporotic men and 40 controls, levels of estradiol, the sex hormone-binding globulin (SHBG), bone formation (OC, BSAP) and bone resorption (ICTP, CTX) were determined [66]. There was no correlation between estradiol and spinal BMD, and only weak correlation to femoral neck BMD. However, SHBG was significantly increased in the osteoporotic individuals compared to controls ($p<0.01$) and negatively correlated to BMD at the femoral neck ($r = -0.37$, $p<0.01$). SHBG also correlated to sCTX ($r = 0.37$, $p<0.01$), but none of the other bone markers. In another population of 283 healthy, ambulatory men ≤70 years of age, bone formation (OC, BSAP) and bone resorption (sCTX, uCTX, Dpd) were negatively associated to BMD, all significantly at the proximal femur and distal forearm [47]. Serum CTX was highly significant correlated at all sites measured by BMD ($p<0.001$). The same inversely relation between BMD and bone turnover markers (NTx, OC) has also been shown previously by Krall et al. in 1997 in 272 elderly healthy men...
aged 65–87 years [61]. Here, the men in the lowest quartile of NTx or OC were associated with 11% higher femoral neck BMD as compared to men in the highest quartile.

Fracture risk

The clinical complications of osteoporosis are fragility fractures. BMD is a widely used estimate of future fracture risk, but around 33–50% of patients with fragility fractures have BMD values above the diagnostics threshold of osteoporosis (T-score less than \(-2.5 \text{ SD})\) [71, 93]. Increasing number of studies [11, 38, 39, 44, 67] point out that fracture risk is also related to the level of bone turnover reflected by a single or a combination of biomarkers. Woo and colleagues were one of the first groups to investigate biochemical markers as predictors of osteoporotic fractures in elderly subjects [103]. They studied the ability of hydroxyproline to predict fractures in 283 elderly Chinese subjects aged \(\geq 60\) years and concluded that increased levels of this marker may be used as a predictive measure. A limitation in this study was a limited number of fractured subjects (\(n=7\)). Later, Garnero et al. (1996) [38] evaluated markers of bone resorption for prediction of risk for hip fracture in elderly women participating in the EPIDOS study. A total of 7,598 healthy women \(\geq 75\) years old participated, of which 126 sustained hip fractures during the 22-month follow-up period. Urinary NTx, CTX and free D-PYR levels at baseline were compared between subjects with or without fractures at follow-up. Increased levels of bone resorption markers predicted increased incidence of hip fracture, independently of initial bone mass. Women with high CTX or high free D-PYR levels had a 4.8 or 4.1-fold increased risk of a hip fracture, respectively. Another study by Garnero et al. in 1998 [39] pointed out that combining urinary CTX measurements with history of prevalent fractures performs as well as hip BMD measurements when estimating the risk of future hip fractures in elderly women. Another group investigated the utility of urinary CTX, serum OC, and BSAP for long-term prediction of vertebral fracture in 603 postmenopausal osteoporotic women. Baseline values of bone turnover markers correlated inversely and significantly with baseline and follow-up (i.e. 36 months later) measures of spine BMD [11]. It was observed that two sequential measurements of serum OC and urine CTX performed at 3-month intervals in combination with BMD measurements could help identify women with the highest risk to present new vertebral deformities. Women in the lowest quartile of a 3-month change in OC had a 69% decreased risk for a vertebral fracture in the subsequent 36-month period compared to those in the highest quartile.

In the OFELY study, BMD and biochemical markers of bone turnover (OC, BSAP and CTX) were assessed in 671 postmenopausal women. During the 9.1-year follow-up period, a total of 158 incident fractures were recorded in 116 women [96]; 48% of fractures were seen in osteopenic women, 44% in osteoporotic women and 8% in women with normal BMD. In the osteopenic women, low BMD was associated with increased fracture risk with an age-adjusted hazard ratio of 2.5. In addition to BMD, age, prior fractures, and bone turnover markers were also independently associated with an increased risk of fractures. In the whole group of osteopenic women, there was a 5.3-fold increased risk of sustaining a fracture if low BMD combined with a prior fracture or a BSAP level corresponding to the highest quartile. The 10-year probability of fracture was 26% if at least one of the three predictors was present, whereas it was only 6% in women without any of the three predictors.

The ability of urinary OC, serum OC, and TRAcP 5b to predict fracture was assessed in 1,040 elderly women [44] of whom 178 women sustained at least one osteoporotic fracture. Both urinary and serum OC were significantly increased in women with a fracture of any type or with vertebral fracture only compared with women without fracture. TRAcP5b also was able to predict the occurrence of a fracture of any type.

Until now only a few studies have assessed the utility of biomarkers for risk evaluation in men. In a case-control cohort study, Meier and colleagues [67] followed 151 elderly men for 6.3 years, 50 men with incident low-trauma fractures and 100 without fractures. In this analysis, S-ICTP was independently associated with fracture incidence; subjects in the highest quartile of S-ICTP had a 2.8-fold increased risk of fracture compared to those within the lowest quartile.

Collectively, serum or urinary levels of bone turnover markers are independent predictors of fracture risk [16, 33, 38, 44]. However, it has not yet been demonstrated whether biochemical markers can really sum up the information on all determinants of the fracture risk. Therefore, the current recommendation for assessment of fracture risk is combined measurements of BMD and biochemical markers [67].

Utility of biomarkers in biomedical research

In this section, we wish to emphasize the potentials and relative advantages of biochemical markers for establishing optimal doses of novel drug candidates in Phase II studies. We provide illustrative data on how these efficacy parameters behaved in Phase II trials that contributed significantly to the approval and marketing of numerous drugs. Finally, we revisit the utility of biochemical markers for improving patient compliance to long-term use of antiresorptive agents.

a. Biochemical markers for drug development
b. Biochemical markers for monitoring treatment efficacy

Biochemical markers for drug development

The diagnosis of osteoporosis is inherently linked to low bone mass, and thus monitoring of changes in BMD during intervention is the main endpoint of the efficacy of drugs
targeting the treatment of osteoporosis. Ideal dose of a given agent is established in Phase II trials. It is to be emphasized that annual changes in BMD are relatively small both when regarding the spontaneous loss of bone mass in the placebo treated group as well as in the group receiving active medical interventions (typically a few % change). Moreover, BMD measurements have an imprecision of 1–2% when using repetitive measurements [34, 43, 49, 56]. Furthermore, despite BMD being important as a surrogate marker of drug efficacy, the access to DXA can be limited. Consequently, much attention has been given to the search for more simple yet useful surrogate markers over the last two decades. The utility of biochemical markers as a powerful tool for fracture risk prediction has recently been emphasized by the observation that increases in BMD during treatment can provide only partial explanation of the reduction in fracture risk [30].

In clear contrast to the imaging techniques, changes in biochemical markers of bone resorption in serum or urine are markedly larger compared with the imprecision of the assays. As an example, changes observed within the first 3 weeks of treatment with bisphosphonates include decreases in serum CTX in the magnitude of 75% with an average short-term intra-individual coefficient of variation (CV) of 7.9% [22]. As the biochemical markers of bone resorption have a low ‘noise-to-signal’ ratio combined with rapid changes in the biochemical marker in response to treatment, use of biochemical markers may provide critical information about the relative effect of an antiresorptive or anabolic treatment that may be used for optimal dosing of various anti-osteoporotic drugs.

Previous studies have indicated that 3- to 6-month changes in the biochemical markers correlate with the change of BMD over 2 years [45, 46, 81, 82]. Accordingly, biomarker based assessment of various doses of drug candidates could considerably improve Phase II development and decrease costs. Biochemical markers can furthermore assist the elucidation of the relative effects of medical interventions on bone formation and resorption, which is useful when distinguishing between antiresorptive and anabolic properties of drug candidates.

Biochemical markers for monitoring treatment efficacy

The ultimate aim of treatment is prevention of fractures, both vertebral and non-vertebral (hip).

Current treatment of osteoporosis includes several drugs targeted towards inhibition of bone loss by decreasing osteoclast activity. Some of these drugs have in clinical trials been demonstrated to reduce the risk of fracture by approximately 40–50% [90] and the effect is usually seen with changed biomarker concentrations within 3–6 months of treatment and increase in BMD after 24 months. Antiresorptive drugs include hormone replacement treatment (HRT), selective estrogen receptor modulators (SERMs), tibolone, bisphosphonates, calcitonin and strontium renalate. Another compound PTH stimulates bone resorption but may also stimulate bone formation when dosed by intermittently injections. In this section, we shortly summarize some of the largest phase III studies conducted for these drug (Table 1) [8, 10, 13, 18, 20, 31, 32, 64, 70, 75, 83]. From these large studies, we see changes in bone turnover markers are soon as 3 months after drug administration, and see a negative correlation between most treatments and markers. Bone markers assist here in determining the efficacy of the drug in question, which is valuable information for a drug development company and not least for patients. Several studies show that bone turnover markers show correlation to BMD changes following drug administration [1, 14, 26, 73, 84, 85, 87, 101]. These studies all show decrease in bone turnover markers following treatment in populations represented from around the world (Scandinavia, Northern Europe, Japan, North America, Thailand, Australia, etc.). Special attention should be paid to the meta-analysis by Crane et al. [26]. Data on spine BMD and five bone turnover markers (OC, BSAP, uNTx, uCTX, sCTX,) from 85 studies using bisphosphonate treatments were analyzed. Spearman correlations were computed to assess the strength of the associations between markers and BMD changes. Baseline BMD at 6 and 12 months were compared to marker changes at 1, 3 and 6 months and revealed modest to strong associations (r=0.63–0.90). In particular, the association was strong for changes at 3 and 6 months in BSAP, OC and NTx, and at 1, 2 and 6 months for uCTX and sCTX. Interestingly, the strongest correlation was found for the 1-month assessment of sCTX (r=0.90).

Compliance

Compliance is an important issue of long-term therapy of chronic diseases. Trivial inconveniences could impair compliance, especially when the medication is given to treat chronic asymptomatic diseases such as osteoporosis. Strict daily dosing of a drug might cause problems for some patients and may obstruct their compliance, which in turn hampers the long-term efficacy of medication. Although a trivial and most cost-effective way of monitoring compliance is by asking the patients, this may not be a completely objective measure. Very useful information can be added by serial measures of bone markers for monitoring patients. In a study of 200 healthy postmenopausal women with an average age of 63.1 years, serial measurements of serum CTX were performed in patients receiving different dosing regimes of ibandronate mimicking different compliances to the oral treatment [99]. The results illustrated that when patients were monitored by serial measurements of CTX, important information could be obtained. The biomarker measurements can not only inform the physician about the efficacy of the treatment, but can also be used to confront the patient regarding her or his achievements, or the need of more rigorous compliance to ensure maximal benefits. The low sensitivity of BMD measurements is not able to provide such early feedback. This advantage of serial...
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Duration (years)</th>
<th>Participants</th>
<th>Age (years)</th>
<th>Participants</th>
<th>ΔResorption markers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ΔFormation markers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ΔBMD (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fracture reduction</th>
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<td>WHI/Women’s HOPE [13, 64]</td>
<td>0.625 mg CEE+ 2.5 mg MPA</td>
<td>5.6/2</td>
<td>Healthy PM women</td>
<td>63/52</td>
<td>16,608/695</td>
<td>-49.2% (NTx) T&lt;sub&gt;1&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>-35.8% (OC) T&lt;sub&gt;1&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Hip +3.7% Spine +3.46%</td>
<td>Hip -33% Vertebral -35%</td>
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<td>BONE study [31]</td>
<td>Iblandronate 2.5 mg/day</td>
<td>3</td>
<td>Osteoporotic PM women</td>
<td>70</td>
<td>2,946</td>
<td>-65.3% (CTX) T&lt;sub&gt;3&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>-35.8% (OC) T&lt;sub&gt;3&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
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<td>Hip -50% Vertebral -62%</td>
</tr>
<tr>
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<td>Alendronate 5 mg/day</td>
<td>3</td>
<td>PM women with low BMD</td>
<td>71/63</td>
<td>2,027/202</td>
<td>-65% (NTx) T&lt;sub&gt;3&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>-50% (BSAP) T&lt;sub&gt;3&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
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<td>Hip -50% &gt;1 vertebral -90%</td>
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<td>1,226</td>
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<td>-37% (BSAP) T&lt;sub&gt;6mo&lt;/sub&gt; vs T&lt;sub&gt;Pl&lt;/sub&gt;</td>
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<td>Vertebral -49% Non-vertebral -33%</td>
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<td>Osteoporotic PM women</td>
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<td>1,649</td>
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<td>1,637</td>
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<td>+10% (BSAP) T&lt;sub&gt;6mo&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Spine +9.6% T&lt;sub&gt;2&lt;/sub&gt; vs T&lt;sub&gt;Pl&lt;/sub&gt;</td>
<td>1 vertebral -65% &gt;1 vertebral -69% Non-vertebral -40%</td>
</tr>
<tr>
<td>PROOF study [20, 32]</td>
<td>Calcitonin 100–400 IU/day</td>
<td>5</td>
<td>Osteoporotic PM women</td>
<td>68–69</td>
<td>1,255</td>
<td>-12–30% (CTX) T&lt;sub&gt;1&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>-9% (BSAP) T&lt;sub&gt;1&lt;/sub&gt; vs T&lt;sub&gt;pl&lt;/sub&gt;</td>
<td>Spine +1–1.5% T&lt;sub&gt;5&lt;/sub&gt; vs T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Vertebral -33% Non-vertebral -18–36%</td>
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BSAP: bone specific alkaline phosphatase, OC: osteocalcin, Dpyr: deoxypyridine, CTX and NTX: C and N-telopeptide of collagen type I. <sup>a</sup>Partly estimated from graphs T<sub>0</sub>, initial value; T<sub>pl</sub>, placebo values; T<sub>3mo</sub>, T<sub>6mo</sub>, value at 3 and 6 months; T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub>, value at 1, 2 and 3 years
measurements of bone markers over the initial 3-month period is also emphasized by the fact that there is strong correlation between drug-induced responses in bone markers at month 3 and subsequent BMD changes at 24–36 months [7, 19, 29, 37, 41, 46, 81]. While the potentials of biomarkers to serve such purposes seems rationalized by the aforementioned studies, the concept needs practical verification by prospective studies.

Summary

In the present review, we attempted to give an updated list of biomarkers including three recently established (Cat K, OPG, RANKL) and eight already well-established (CTX, ICTP, NTx, PYR, BSAP, OC, PINP/PICP, TRAcP) ones. Biomarkers have become key players in bone-related biomedical research. The strengths of biochemical markers lies within their dynamics that makes it possible to document early responses to interventions, which correlate well with subsequent changes in bone mass and fracture risk. The development of medical drugs for the treatment of osteoporosis is an expensive process, which at present time demands the participation of hundreds (Phase II) or thousands (Phase III) of patients in trials run for at least 2 years. Biochemical markers are widely used in in vivo, ex vivo and in vitro experiments [92].

The application of biochemical bone markers should be based on careful consideration as to which metabolic events are in need of assessment. The markers reviewed here are all available in test formats having acceptable technical performance, and the selection of any specific marker should therefore be based on a clear understanding of the metabolic events leading to the generation of the analyte. In clinical and epidemiological studies of osteoporosis and other metabolic bone diseases, bone resorption and bone formation is often assessed on the basis of measurements of serum samples, as the marker levels here do not have to be corrected for creatinine. Serum CTX-I in combination with either PINP or OC seems a reasonable choice as they reflect the degradation and synthesis of matrix molecules that are very abundant in the skeleton. Consequently, these markers have been used in numerous studies, some of which have been referenced here. Further studies are needed to gain more experience with what advantages we can gain by combining collagenous resorption markers with the different non-collagenous osteoclast markers that provide insights into changes of osteoclast number under different pathophysiological processes or during treatment with antisresorptive or anabolic drugs.

However, it is still an ongoing debate, whether biomarkers combined with BMD measurements can be primary end-points of drug evaluation. If the answer to this question is yes, we will be able to lower the costs of drug-development, and provide patients with less expensive medications for the prevention and treatment of osteoporosis.

Conflict of interest Diana J Leeming, Morten A. Karsdal and Per Qvist are employed by Nordic Bioscience A/S, a company engaged in the development and marketing of bone and cartilage markers.

References


APPENDIX 2
Biochemical approach to the detection and monitoring of metastatic bone disease: What do we know and what questions need answers?

László B. Tankó · Morten A. Karsdal · Claus Christiansen · Diana J. Leeming

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Abstract Metastatic spread to bones frequently occurs in several types of cancer diseases, in particular breast, prostate, and lung cancer. Infiltration of bone by tumour cells is a source of several complications including severe bone pain, spinal cord compression, hypercalcemia, pathologic fractures, all reducing quality of life and worsening prognosis. Therefore, early recognition of bone metastases is among the highest priorities in the clinical management of cancer disease. Currently, detection and staging relies on radiological imaging techniques (scintigraphy, radiography, computer tomography, etc.). Due to their limited sensitivity and/or inconveniences, irradiation, and considerable costs related to serial use, they are not suited for close monitoring of cancer patients to capture skeletal spread in an early stage or to follow-up on therapeutical responses. Interaction of tumour cells with surrounding bone cells leads to enhanced bone resorption and/or bone formation. These cellular processes result in the release of numerous epitopes that, if detected by immunoassays, can reflect the changes of the rate of bone turnover and the occurrence of metastatic spread to bone. Numerous studies reported elevated levels of bone turnover markers in patients with bone metastases proportionally to the extent of skeletal involvement. Furthermore, preliminary data suggest that biomarkers can predict skeletal-related events (SREs), disease progression, and even cancer-related death. The present review intends to summarize the list of emerged biomarkers, major studies assessing their relative utility for detection of bone metastases in different types of cancer disease, and discuss their potentials for becoming part of screening protocols for improving our success rate in the early detection of metastatic bone disease.

Keywords Bone turnover · Biochemical markers · Bone metastases · Detection · Cancer patients · Diagnostic

1 The rational for a biochemical approach

Bone tissue is under continuous renewal owing to two opposite activities of bone cells, the bone resorbing osteoclasts and the bone forming osteoblasts. These processes are normally tightly coupled in time and space, meaning that old bone resorbed by osteoclasts is rapidly replaced by the same amount of new bone formed by osteoblasts. Alteration of this tightly regulated balance leads to uncoupling and a net event of accelerated bone loss or increased bone formation. Whereas these changes affect the total skeleton in metabolic bone diseases, in metastatic bone disease (MBD) they occur only at sites of tumour implantation. Bone lesions can be either osteolytic or osteogenic depending on which cells are stimulated predominantly by tumour cells.
**Focal osteolysis** Activation of osteoclasts by tumour cells is required to facilitate expansion of the metastasis in the mineralized matrix. Bone resorption around metastatic foci is predominantly mediated by osteoclasts. Osteoclast differentiation and activation are regulated at the local level by the relative expression of receptor activator of nuclear factor-κB (RANKL) and osteoproteregin (OPG) [1]. RANKL and OPG are mainly produced by the osteoblast lineage. RANKL acts directly on osteoclast precursors and mature osteoclasts through its receptor RANK to increase osteoclast differentiation and activation. OPG is a decoy receptor for RANK [2]. The relative expression of RANKL and OPG is modulated by proinflammatory cytokines (TNFα, TNFβ, IL-1, IL-6) and eicosanoids released by tumour cells [3]. Tumour cells also can produce PTHrP, which has been shown able to increase RANKL and decrease OPG expression in stromal cells and thus is a particularly important player in the development of osteolytic lesions in MBD [4].

**Focal osteogenesis** Prostate cancer is by far the most common neoplasm that produces this reaction in bone tissue. A number of growth factors have been identified including insulin-like growth factors (IGFs), fibroblasts growth factors (FGF), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), and members of the morphogenetic protein family [5, 6]. Osteoblastic metastases can be caused by tumour-secreted endothelin-1 (ET-1) [7]. Other probable contributors include the NH₂-terminal fragment of the serine protease and urinary plasminogen activator (uPA) [5]. Nevertheless, the major mediator of focal osteogenesis remains to be identified.

Figure 1 is a schematic diagram illustrating the interactions between tumour cells, osteoblasts, and osteoclasts resulting in the release of biomarkers. The rate of formation of bone matrix can be assessed by measuring a prominent enzymatic activity or generated matrix components whereas the rate of resorption can be quantified by measuring different peptide fragments released during the degradation of type I collagen molecules. Due to coupling between these events, biomarkers of formation and resorption can be elevated simultaneously, reflecting the overall rate of bone turnover. It is important to emphasize that bone turnover markers merely reflect the consequences of the interaction between an invasive and hormonally active malign tissue and the surrounding bone cells, and hence they are not able to reflect the primary disease, i.e., the type of cancer. Another point worth emphasis is that at sites of metastatic

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**Fig. 1** Interactions between tumour cells, osteoblasts, and osteoclasts in the proximity of a bone metastasis. Tumour cells may stimulate bone cells with predominantly osteolytic or osteoblastic mediators. Increased activity of osteoblasts and osteoclasts results in the release of different biomarkers (enzymes, bone matrix components, and degradation peptides from collagen type I), which can be detected in the serum/plasma and/or urine. At sites of bone metastases, bone remodelling is accelerated maintaining bone tissue and hence collagen molecules in an immature state. Therefore, biomarkers such as non-isomerized cross-linked C-terminal telopeptide of collagen type I (ααCTX) that able to reflect the source (i.e., young bone) represent a promising marker for detecting changes in bone turnover attributable to metastatic bone disease.
tumour infiltration, the markedly accelerated bone turnover does not allow sufficient time for the newly formed bone matrix to mature before it undergoes resorption again. Therefore, degradation peptides that are specific for immature collagen molecules can be useful and likely sensitive indicators of MBD.

The present review was sought to summarize biomarkers that have emerged so far and major studies demonstrating their relative utility for detection of bone metastases. Preference will be given to studies comparing several biomarkers in a patient population representing lung, prostate and breast cancer. Finally, we attempt to outline the remaining questions that need answers before we can make general recommendations to the use of biomarkers in daily clinical practice.

2 Proposed biomarkers

2.1 Bone formation markers

2.1.1 Osteocalcin (OC)

Osteocalcin (former Gla-protein) is synthesized and secreted by osteoblasts and constitutes the major non-collagenous protein of bone matrix (Watts 1999), but a fraction is released into the circulation where it can be detected by immunoassay [8]. The intact molecule represents approximately a third of the immunoreactivity in the adult serum or plasma. Another third is represented by several small fragments and the rest by a large N-terminal mid-molecule fragment of 43 amino acids [9]. Measuring both the intact and the N-mid fragment with appropriate antibodies results in the most robust and sensitive OC assay [10]. Intra- and inter-assay for OC assays have been reported to be <6.4 and <6.1%, respectively [11].

2.1.2 Bone-specific alkaline phosphatase (BSAP)

In osteoblast cultures that enter the mineralization stage, all cells become BSAP positive, which emphasizes the involvement of this enzyme in bone formation. Serum BSAP concentration was shown to have a linear relationship with osteoblast and osteoblastic precursor activity [12]. Serum BSAP can be assessed indirectly after precipitation with lectin. The monoclonal antibody-based immunoassay has demonstrated intra- and inter-assay CVs of <4 and <10%, respectively [13].

2.1.3 Procollagen I extension peptides: PICP and PINP

During the extracellular processing of collagen I, there is a cleavage of the N-terminal and C-terminal extension peptides before fibrils are fully formed. The thereby formed PINP and PICP peptides can be detected in serum/plasma by specific immunoassays [9]. The intra- and inter-assay variation for a PINP ELISA was 4.6–5.3 and 2.9–4–9% [14], respectively. For a PICP ELISA these parameters were 5–7 and 5–7%, respectively (Metra CICP, specifications, 2006).

2.2 Bone resorption markers

2.2.1 C-terminal telopeptide of collagen type I (CTX-I)

CTX-I is an eight-amino-acid peptide fragment which is generated by cathepsin K activity during osteoclastic bone resorption. The CTX epitope contains an aspartyl-glycine motif (DG) that is prone to spontaneous isomerization during maturation of collagen [15]. In other words, EKAHD(α)GGR epitopes are released during degradation of newly synthesized type I collagen (young bone), whereas EKAHD(β)GGR epitopes are released from matured collagen type I (old bone). Resorption rate of newly synthesized collagen type I can be assessed by specific immunoassays targeting the detection of cross-linked non-isomerized ααCTX in urine samples. Degradation rate of matured collagen can be estimated by another specific assay targeting cross-linked isomerized ββCTX in both urine and serum samples. The intra- and inter-assay CV for these assays are <9% [14].

2.2.2 C-terminal telopeptide of collagen type I (ICTP)

ICTP is a relatively large hydrophobic phenylalanine-rich pyridinolines cross-link between the two α1 chains in the C-terminal telopeptide of matured collagen type I [16]. The ICTP epitope neighbours CTX in the C-terminal peptide but it is released as a result of matrix metalloproteinase activity [17]. In this case, Cathepsin K activity eradicates the ICTP epitope [18]. ICTP can be measured in serum and plasma by a specific radioimmunoassay with intra- and inter-assay CV of 3 and 8%, respectively [16].

2.2.3 N-terminal telopeptide of collagen type I (NTX)

NTX is an eight-amino-acid epitope (JYDGKGVG), which is also cleaved by osteoclastic cathepsin K activity. NTX can be measured in serum and urine by a specific immunoassay. The intra- and inter-assay variations are 6.1 and 4.0%, respectively [19].

2.2.4 Deoxypyridinoline (D-Pyr) and pyridinolines (Pyr)

Pyr is widely distributed in collagen type I of bone, collagen type II of cartilage, and in smaller amounts in other connective tissues (except for skin). D-Pyr is not
specific for bone matrix, but large amounts can only be found in bone collagen. These cross-links are generated during the late phase of fibril formation, and hence characterize the degradation of mature collagen [9]. These degradation products are detectable in both serum and urine by specific immunoassays. The intra- and inter-assay CVs have been reported to be 4–8 and 3–5%, respectively (Metra DPD, specifications, 2006).

2.3 Osteoclast regulatory proteins

2.3.1 Receptor activator of nuclear factor NF-κB ligand (RANKL)

It is the main mediator of osteoclast activation, differentiation, and survival [20]. RANKL is a member of the tumour necrosis factor (TNF) family and is produced by bone-forming osteoblasts and activated T lymphocytes. Circulating RANKL can be measured by specific immunoassay with intra- and inter-assay variation of 2.2 and 8.2%, respectively (Immundiagnostik, total sRANKL manual 2004).

2.3.2 Osteoprotegerin (OPG)

OPG is a soluble decoy-receptor, which is produced in different tissues e.g., bone, liver, stomach, intestine and lung. Osteoblasts secrete OPG that binds to RANKL and thereby inhibit the regulatory effect of RANKL on osteoclast activation and proliferation [21]. Intra- and inter-assay variation for an available OPG assay is <10% (Immundiagnostik, Osteoprotegerin ELISA manual, 2004).

2.3.3 Tartrate-resistant acid phosphatase (TRACP 5b)

TRACP is a glycoprotein produced in mature osteoclasts, activated macrophages and dendritic cells. The polypeptide chain of TRACP is cleaved by proteases into two isoforms 5a and 5b, which activates phosphatase activity [22]. The isoform TRACP 5b is derived from osteoclasts and has been proposed to reflect osteoclast number rather than bone resorption [23, 24]. TRACP 5b can be measured in serum samples with intra- and inter-assay CVs of 2.1–7.9 and 4.9–13%, respectively [22].

3 Frequency of metastases in different cancer types

The worldwide incidence of skeletal complications associated with malign diseases is more than 1.5 million every year. Patients at highest long-term risk are those with breast (65–70%), prostate (65–75%), and lung (40%) cancer as primary tumours [25]. Prostate cancer give rise to osteoblastic, lung cancer to osteoclastic, whereas breast cancer osteolytic or mixed lesions [26].

4 Utility of biomarkers

The questions that arise when discussing the utility of biomarkers of bone turnover in the clinical management of bone metastases are as follows:

(a) Can these different biomarkers differentiate cancer patients with or without metastases?
(b) Can biomarkers reflect the severity of MBD?
(c) Can biomarkers reflect the osteoclastic or osteoblastic nature of bone metastases?
(d) Can biomarkers predict disease progression and adverse clinical outcomes?
(e) Can biomarkers replace imaging techniques in the early detection of MBD?

4.1 Can biomarkers differentiate cancer patients with or without bone metastases?

In a recent study from our laboratory that included breast, prostate, or lung cancer patients with or without MBD (n= 161), we assessed four different collagenous markers of bone resorption, a marker of bone formation, an indicator of osteoclast number, and two osteoclast regulatory proteins for their ability to differentiate cancer patients with or without MBD. This study allowed assessment of the relative utility of the different markers in different types of cancer disease within the same study population [12]. The αα-CTX, NTX, and ICTP markers were all indicative for the presence of bone metastases regardless of cancer type. However, differences between subjects with or without MBD were more pronounced in breast and prostate cancer patients (p<0.001) than in lung cancer patients (p<0.05). ββ-CTX was able to point out the presence of MBD in prostate and breast cancer, but not in lung cancer patients. The generally higher sensitivity of collagenous resorption markers for the diagnosis of bone metastases is supported by observations by several other groups ([26], in patients with all three types of cancer; [27], in patients with lung cancer; [28], in patients with prostate cancer).

In our study, presence of MBD was accompanied by significantly increased BSAP levels in breast cancer and in particular prostate cancer patients (p<0.001). The formation marker however was unable to discriminate lung cancer patients with or without MBD. A comparative study by Gamero et al. [28] undertaken in prostate cancer patients showed that other formation markers, PICP and OC were poorer indicators of MBD than BSAP. The relative increases in these three markers in patients with MBD...
were 67, 79, and 138%, respectively. The modest diagnostic value of PICP for detection of MBD in prostate cancer patients is also underscored by Fukumitsu et al. [29]. Studies on lung cancer patients also conclude poor diagnostic value of OC and PICP compared with BSAP [27]. In contrast, in a study on breast cancer patients, PINP was able to discriminate patients with or without bone metastases, and serum concentrations of the biomarker correlated with osseous spread in terms of number and size of the bone lesions [30]. Nevertheless, the diagnostic sensitivity was relatively low (50%). Findings by Chrapko et al. [31] in cancer patients with different severity of MBD (Group I: no hot spots, Group II: up to ten spots, and Group III: more than ten spots on scintigraphy), PINP was significantly elevated in advanced MBD only. Collectively, of the different bone formation markers, BSAP seems to be the most useful biomarker for detection of bone metastases, in particular in prostate cancer patients.

As mentioned earlier, the number of osteoclasts increases in the proximity of bone metastases. In our study, TRAcP 5b was highly indicative for the presence of skeletal involvement in both breast cancer and prostate cancer patients, though not in lung cancer patients. Similar findings were reported by Lyubimova et al. [32] who found relatively high diagnostic specificity and sensitivity of this biomarker in patients with prostate cancer (83 and 71%) as well as in breast cancer patients (87 and 82%). In the study by Koizumi et al. [33], TRAcP 5b was a more sensitive indicator of MBD than NTx, which was only increased in patients with advanced skeletal spread. Collectively, these findings inspire further large-scale evaluation of TRAcP 5b alone or in combination with resorption markers for monitoring cancer patients.

Regarding the utility of osteoclast regulatory proteins, the literature is somewhat contradictory. In the presence of MBD, OPG was significantly elevated in breast cancer but not in prostate or lung cancer patients. In contrast, Jung et al. [34] found OPG to be the best indicator of bone metastases in prostate cancer patients when comparing the relative utility of ten different biomarkers (total alkaline phosphatase, BSAP, PINP, CTX, NTX, TRAcP 5b, bone sialoprotein, OC, OPG, and RANKL). Their logistic regression analysis resulted in a model with OPG and TRAcP as variables that predicted bone metastases with an overall correct classification of 93%. Further studies are needed to clarify whether the diagnostic value of OPG is specific for prostate cancer.

In our study, RANKL was not elevated in patients with MBD compared to those without and this finding was applicable to all cancer types. In prostate cancer patients, similar results were found by Jung et al. [35]. Based on these findings, RANKL is unlikely to emerge as a useful biomarker to assist early diagnosis of MBD.

4.2 Can biomarkers reflect the severity of MBD in cancer patients?

We investigated the relationship between skeletal tumour load and elevations in biomarker levels in serum or urine in a pooled group of breast and prostate cancer patients [12]. We found strong linear associations between these parameters for most biomarkers (p<0.001), except for OPG and RANKL. Importantly, all markers were significantly elevated in patients with one to four metastases (Soloway 1), this finding being most consistent for NTx and ααCTX. In a study including lung cancer patients only, similar linear associations were found between the number of bone metastases and biomarker levels (total alkaline phosphatase, BSAP, PYR, D-PYR, and ICTP) [36]. Oremek et al. [37] showed this relationship for CTX and BALP in a large group of cancer patients (prostate, colon, breast, liver and pancreas cancer), whereas Demers et al. [38] for NTx and BSAP in 77 cancer patients (52 being breast cancer). Thus, these findings provide evidence for the potentials of biochemical markers to reflect not only the presence but also the extent of skeletal invasion.

As indicated by Fig. 2, the relative increases due to the presence of bone metastases were most pronounced for ααCTX followed by BSAP and NTX. This finding is supported by observations in prostate cancer patients showing that of seven different biomarkers, ααCTX revealed the largest relative elevations to MBD [28].
higher sensitivity of ααCTX could be explained by the fact that this epitope arises at sites of high bone remodelling, where collagen fibrils do not have time to mature and undergo β-isomerization [39].

4.3 Can absolute level of biomarkers indicate the osteoblastic or osteolytic nature of metastatic bone lesions?

Although the diagnostic role of biomarkers in discriminating osteoblastic and osteolytic metastases is of secondary importance with reference to the information provided by imaging techniques, observations by Demers et al. indicate that the absolute concentration of BSAP and NTx in patients with sclerotic metastases is much higher than in patients with osteolytic or mixed typed lesions [38].

4.4 Can biomarkers carry predictive value and warn clinicians regarding the risks of disease progression and future skeletal complications?

The first prospective data concerning the predictive value of NTx for the risk of skeletal-related events (SRE) in patients with MBD was published by Brown et al. in [40]. They measured this biomarker on a monthly basis in 121 cancer patients (91 having breast cancer) and related to the incidence of SRE over a 6-month period. Patients with an increased NTx level (≥100 nmol/mmol creatinine) were 19 times more likely to develop a SRE during the first 3 months than those with low levels (<100 nmol/mmol creatinine).

The same group further assessed the same biomarkers in a larger trial including patients with either prostate cancer (n=203) or other solid tumours, mainly non-small cell cancer of the lung (n=238) [41]. The biomarkers were measured every third month to assess the predictive value of both baseline and on-study measurements. Patients with high levels of NTx (≥100 nmol/mmol creatinine) or BSAP (≥146 IU/L) at baseline had a significantly higher incidence of SRE (i.e., radiotherapy or surgery to bone, pathologic fractures, spinal cord compression, or change in antineoplastic therapy) than those with low levels. NTx was notably better to discriminate patients with poor or good prognosis as measured by the median survival time. In terms of relative risk, patients with increased NTx at baseline had increased risk for a SRE, shorter time to first event, disease progression, and death compared with those with low NTx levels (Table 1). When the on-study levels of NTx were used for the analysis, the relative risks were even higher. High BSAP levels at baseline also were accompanied with increased risk for SREs, shorter time to first event, disease progression, and death, these correlations being stronger when using on-study levels for the analysis. The authors concluded that NTx levels were more consistent prognostic indicators than BSAP for all tumour types. Part of the explanation is that high BSAP levels can have not only negative but also positive prognostic implications (e.g., bone formation to repair lesions during therapy).

The third report comes from a retrospective analysis of data from clinical trials including cancer patients with MBD who received treatment with bisphosphonates (n=1,824) [42]. Of these, 1,462 patients were treated with zoledronic acid (490 breast cancer, 411 prostate cancer, 210 multiple myeloma, 183 non-small cell lung cancer, and 168 other tumours), and 362 patients were treated with pamidronate (254 breast cancer and 108 multiple myeloma). High (≥100 nmol/mmol creatinine) and moderate (50–99 nmol/mmol creatinine) levels of NTx but to some extent also high levels of BSAP (≥146 U/L) were predictive for future clinical outcomes including SREs, first SRE, disease progression, and death. The relative risks associated with

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Skeletal related events (SRE): pathologic fracture (n=143), radiotherapy to bone (n=240), surgery to bone (n=24), spinal cord compression (n=29), change in antineoplastic therapy (n=18) – total number of events (n=454).
high baseline levels of the two biomarkers are summarized in Table 2. The relationships between Ntx levels and outcome were broadly similar, irrespective of the underlying tumour type. Patients who developed or maintained high Ntx or BAP levels during the course of zoledronic acid therapy were most at risk for deterioration in skeletal integrity, SREs, and a poor clinical outcome. On other words, recent (<6 months old) marker assessments had even greater predictive value than the baseline assessment at later time points during the trial. The authors expressed enthusiasm about the prognostic implications of NTx assessments, which they believe could become broadly applicable in the oncology setting regardless of the primary malignancy.

Collectively, this new data suggest that serial measurements at regular intervals during the course of MBD could provide useful information regarding which patient should be the subject of further diagnostic examinations and early interventions to prevent the burden caused by skeletal complications. However, these findings need confirmation by independent groups before general recommendations can be issued for the medical community.

4.5 Can biomarkers replace bone scintigraphy in the early detection of bone metastases?

Bomabardieri et al. [43] examined 149 breast cancer patients, 33 of which had bone metastases. They measured numerous biomarkers including two formation markers (OC and BSAP) and two resorption markers (CTX and ICTP). In multivariate analysis, menopausal status and bone metastases were independent contributors to the variation in biomarker levels. BSAP was the best to discriminate scan-positive from scan-negative patients closely followed by ICTP. Combination of the two latter markers further improved the diagnostic value, yet the overall discrimination was labelled as poor. To achieve 95% specificity, sensitivity dropped to 20%, whereas when achieving 95% sensitivity, the specificity was below 10%.

In a relatively small study, Pectasides et al. [44] assessed the diagnostic specificity and sensitivity of NTx and BSAP for detection of skeletal metastases in patients with breast cancer. Both markers were significantly higher in patients with skeletal metastases compared with those without. Using a cut-off value of 29.7 nmol/L for NTx, specificity and sensitivity were 87.1 and 45.5%, respectively. For BSAP (cut-off value 50.6 U/L), the respective numbers were 90.3 and 54.5%. When focusing on patients who were not receiving hormone therapy, the specificity and sensitivity considerably enhanced, particularly for BSAP (92.3%, 70.6%—cut-off value 50.0 U/L).

Ebert et al. [36] asked a similar question for TALP, BSAP, PINP, PICP, PYR, D-PYR, ICTP, CTX (β-Cross-Laps), and TRAcP 5b in a population of 49 lung cancer patients with skeletal metastases, 83 lung cancer patients without metastases, 12 patients with benign lung diseases,
and 18 healthy subjects. The sensitivity and specificity of bone scintigraphy were 100 and 76.4%, respectively. Positive predictive value was 70%, whereas the negative predictive value was 100%. Using cut-off values that correspond to 95% specificity in the group of healthy subjects, the sensitivity of different marker assays were (specificity in parenthesis): TALP 33.3% (97.5%), BSAP 22% (100%), PINP 18.4% (97.5%), PICP 2.1% (95.2%), PYR 91.8% (24.1%), D-PYR 83.7% (24.1%), ICTP 75.5% (44.6%), CTx 45.8% (77.5%), and TRAcP 5b 14% (84%).

The corresponding data for diagnostic efficiency were as follows: TALP 73.6%, BSAP 77.1%, PINP 67.7%, PICP 61.1%, PYD 48.5%, D-PYR 55.2%, ICTP 56.1%, CTx 65.6%, and TRAcP 5b 58.7%, respectively. The positive predictive value ranged from 20% (PICP) to 100% (BSAP), whereas the negative value from 62.7% (PICP) to 84% (PYR). In the ROC analysis, TALP, followed by BSAP, PINP, and PYR performed the best.

- The bone formation marker BSAP can be of useful assistance in the diagnostic of MBD in prostate cancer patients.
- Preliminary experience with NTx and BSAP indicate that biomarkers may predict SREs, disease progression, and death, which need to be confirmed by independent groups and tested with other promising markers.
- Although biomarkers alone may unlikely to totally replace imaging techniques in the clinical management of cancer patients, the current data seem to nurture the notion that monitoring of biomarkers of bone turnover in cancer patients at regular intervals might increase our success rate of capturing MBD in its early state.

6 Key unanswered questions

Prospective studies are awaited to clarify the following questions:

- Can elevation of a biomarker or combination of biomarkers become reliable indicator of spread to bone at an early stage and hence an indicator of need to examine the patient with advanced imaging techniques?
- Which biomarker or combinations of biomarkers offers the highest sensitivity and specificity (head-to-head comparison is needed)?
- How ααCTX compared with NTx and BSAP performs in prospective studies for detection of skeletal spread and skeletal-related events?

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### Incorporation by reference:

where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.

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| IX-3 | Claims | 21 | ✓ |
| IX-4 | Abstract | 1 | ✓ |
| IX-5 | Drawings | 15 | ✓ |
| IX-7a | Sub-total number of sheets | 168 |  |
| IX-6a | Sequence listing part of description | 610 | ✓ |

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778

### IX-8 Accompanying Items

| IX-8 | Fee calculation sheet | paper document(s) attached | electronic file(s) attached |
| IX-18 | PCT-SAFE physical media | - | ✓ |

### IX-20 Table of the drawings which should accompany the abstract

| IX-21 | Language of filing of the international application | English |

### X-1 Signature of applicant, agent or common representative

| X-1-1 | Name (LAST, First) |
| X-1-2 | Name of signatory |
| X-1-3 | Capacity |

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| 10-2 | Drawings: |
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| 10-2-2 | Not received |
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| 10-4 | Date of timely receipt of the required corrections under PCT Article 11(2) |
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<tr>
<td>PCT application number</td>
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<td>Nordic Bioscience A/S</td>
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<td>Number of applicants</td>
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<tr>
<td>Country</td>
<td>DK</td>
</tr>
<tr>
<td>Title</td>
<td>Fibrosis Biomarker Assay</td>
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Documents submitted:
- eolf-pkda.xml
- eolf-appb.xml
- eolf-fees.xml
- pct101.GML
- eolf-appb-P000002.pdf (21 p.)
- eolf-appb-P000003.pdf (1 p.)
- eolf-appb-P000004.pdf (15 p.)
- eolf-abst.txt
- pct101.1WO
- referenc.inf
- eolf-requ.xml
- eolf-seql.txt
- eolf-vlog.xml
- eolf-appb-P000001.pdf (126 p.)
- pct101u.gml

Submitted by: CN=P. Smart 13516, O=Beck Greener, C=UK
Correction by the EPO of errors in debit instructions filed by eOLF

Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 if ADA, Supplement to OJ EPO 10/2007).

/European Patent Office/
Fibrosis Biomarker Assay

The present invention relates to assays for biomarkers useful in the diagnosis of fibrosis disease and prognosis of its development, including biomarkers indicative of the risk of developing fibrosis after a chronic injury.

In particular, according to the present invention, biomarkers relating to degradation fragments of Collagen type I, III, IV, V, and VI, elastin, C-reactive protein, and proteoglycans including Biglycan, Decorin, Versican, and Perlecan are found to be useful.

Fibrotic diseases (including those listed in Table 1) are a leading cause of morbidity and mortality, e.g. cirrhosis with 800,000 death per year worldwide.

Table 1. Different fibrotic diseases

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Examples of Causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Viral hepatitis</td>
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<tr>
<td></td>
<td>Schistosomiasis</td>
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<tr>
<td></td>
<td>Steatohepatitis (Alcoholic or non-alcoholic)</td>
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<tr>
<td>Lung</td>
<td>Idiopathic pulmonary fibrosis (IPF)</td>
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<tr>
<td></td>
<td>Systemic sclerosis (Scleroderma)</td>
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<tr>
<td>Kidney</td>
<td>Nephrogenic systemic fibrosis (NSF)</td>
</tr>
<tr>
<td></td>
<td>Diabetes</td>
</tr>
<tr>
<td></td>
<td>Untreated hypertension</td>
</tr>
<tr>
<td>Heart</td>
<td>Heart attack</td>
</tr>
<tr>
<td></td>
<td>Hypertension</td>
</tr>
<tr>
<td></td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td></td>
<td>Restenosis</td>
</tr>
<tr>
<td>Eye</td>
<td>Macular degeneration, retinal and vitreal retinopathy</td>
</tr>
<tr>
<td>Skin</td>
<td>Systemic sclerosis and scleroderma, keloids, hypertrophic scars, burns, genetic factors NFS</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Autoimmune/hereditary causes</td>
</tr>
<tr>
<td>Intestine</td>
<td>Crohn’s disease/inflammatory bowel disease</td>
</tr>
</tbody>
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