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PERSPECTIVES

New Biochemical Markers of Bone Turnover

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Abstract

Current biological markers of bone turnover have proven useful in improving fracture risk assessment and monitoring treatment efficacy in postmenopausal osteoporosis. Progress in the characterization of important biological pathways regulating bone cell activity and the organic components of bone matrix has led to the development of new biochemical markers. These include the non-collagenous protein periostin, resorption-mediated osteocalcin fragments, the osteoclastic enzymes tartrate-resistant acid phosphatase and cathepsin K, the regulators of osteoclastic (OPG, RANKL) and osteoblastic (Wnt signaling molecules) activity and the post-translational modifications of matrix molecules. One of the most interesting developments has been the demonstration that the non-enzymatic modifications of bone collagen, including glycation-mediated crosslinks and the isomerization of aspartic acid, contribute to fracture resistance independent of bone mineral density (BMD). Preliminary clinical studies have shown that increased systemic levels of the glycated crosslink pentosidine and the urinary ratio between native (α CTX) and isomerized (β CTX) type I collagen C telopeptide are associated with fracture risk in postmenopausal women independent of BMD and may respond differently to the various anti-resorptive therapies. The identification of bone-specific post-translational modifications of bone matrix proteins that influence bone fracture resistance should lead to the development of new biological markers that will be useful in assessing the contribution of changes in bone matrix properties to fracture risk during aging, disease and treatment. *IBMS BoneKEy*. 2008 March;5(3):84-102.

Keywords: Bone markers; Collagen; Osteoporosis; Pentosidine; Fracture

Introduction

Although clinical assessment of bone mineral density (BMD) by dual X-ray absorptiometry, which largely reflects the mineral phase of bone tissue, is the current gold standard for the diagnosis of osteoporosis, about 50% of postmenopausal women with incident fracture have BMD levels above the WHO criteria for osteoporosis (1;2), indicating that factors not reflected in BMD measurement contribute to bone fracture resistance. Indeed, bone fragility also depends on the morphology and architecture of bone as well as on the material properties of bone matrix that cannot be readily assessed. In osteoporosis, low BMD levels and the microarchitectural alterations of bone tissue are both related to abnormalities of bone turnover. Consequently, it has been suggested that bone fracture resistance may be reflected in part, independent of BMD levels, by measuring bone turnover using specific serum and urinary markers of bone formation and resorption. This *Perspective* will examine recent developments in biochemical markers of bone turnover.

Current Biochemical Markers of Bone Turnover

Bone remodeling is the result of two opposite activities: the production of new bone matrix by osteoblasts and the destruction of old bone by osteoclasts. The rates of bone production and destruction can be evaluated either by measuring predominantly osteoblastic or osteoclastic enzyme activities or by assaying bone matrix components released in the bloodstream and excreted in the urine. These have been separated into markers of formation and resorption, but it should be kept in mind that in disease states where both events are coupled and change in the same direction, such as in osteoporosis, any

marker will reflect the overall rate of bone turnover.

At present, in postmenopausal osteoporosis the most sensitive markers for bone formation are serum total osteocalcin, bone alkaline phosphatase (bone ALP), and the procollagen type I N-terminal propeptide (PINP) (3). For the evaluation of bone resorption, most assays are based on the detection in serum or urine of type I collagen fragments, which account for the vast majority of the organic phase of bone tissue. These include the enzymatic telopeptide crosslinking molecules pyridinoline (PYD) and deoxypyridinoline (DPD), the cathepsin K (CTX, NTX) and matrix-metalloprotease (MMP)-generated telopeptide peptides (CTX-MMP or ICTP) and fragments of the helical portion (helical peptide) (3;4). The measurements of ICTP may be particularly useful in the assessment of pathological bone resorption observed in metastatic bone diseases (5) or rheumatoid arthritis (6), whereas cathepsin K-derived markers (CTX and NTX) are more sensitive in postmenopausal osteoporosis. The measurements of most of these biochemical markers can now be achieved with high throughput and analytical precision on automated platforms (7).

New Biochemical Markers of Bone Metabolism

Currently available biological markers have been useful for the investigation of the pathophysiological processes of metabolic bone diseases and drug development in postmenopausal osteoporosis and metastatic bone disease (8), and guidelines for their use in the management of individual patients are becoming available (9-11). However, they do have some limitations. Current biochemical markers of bone resorption are based primarily on type I collagen, which is not bone-specific and is widely distributed in several tissues of the body. Some type I collagen-based bone resorption markers are characterized by significant intra-patient variability, which impairs their use in individual patients. The systemic levels of biochemical markers reflect global skeletal turnover and do not provide distinct information on the remodeling of different bone envelopes, *i.e.,* trabecular, cortical and periosteal, although their relative contribution may vary with aging, disease and treatment. Finally, current markers mostly reflect quantitative changes of bone turnover and do not provide information on the structural abnormalities of bone matrix properties, which are an important determinant of bone fragility, especially toughness. Recently, new biochemical markers have been investigated to address some of these limitations (Table 1).

Non-Collagenous Bone Proteins and Osteocalcin Fragments

Although the vast majority of bone matrix is composed of type I collagen molecules, about 10% of the organic phase is comprised of non-collagenous proteins, some of which being specific for bone tissue. It has been suggested that measurements of these proteins or fragments thereof could constitute specific biochemical markers of bone turnover.

Bone sialoprotein (BSP) is an acidic, phosphorylated glycoprotein of 33 kDa (glycosylated: 70–80 kDa) that contains an RGD integrin binding site. Although BSP is relatively restricted to bone, it is also expressed by trophoblasts and is strongly upregulated in a variety of human primary cancers, particularly those that metastasize to the skeleton, including breast, prostate and lung cancer (12). A recent case-control, retrospective study has shown that high expression of BSP by non-small-cell lung tumor tissue was strongly associated with the development of bone – but not soft tissue – metastases (13). A small amount of BSP is released in the circulation and as such is a potential marker of bone turnover (14). A high serum level of BSP has been shown to be associated with the progression of prostate cancer and with the development of bone metastases in patients with localized breast carcinoma (15). Serum BSP levels have also been reported to be increased in malignant bone disease and are decreased by bisphosphonate treatment in patients with bone metastases and Paget's disease (14).

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Non-collagenous proteins of bone matrix and fragments	Osteoclastic enzymes	Regulators of osteoclast-osteoblast differentiation/activity	Markers of bone matrix properties
- Bone sialoprotein - Osteopontin - Periostin - Urinary mid-molecule osteocalcin fragments	TRAPC5b - Cathepsin K	-OPG/RANK-L (osteoclast) -Wnt signaling molecules (Dkk1/sFRP) -Sclerostin (osteoblast)	- Non enzymatic glycation-mediated modifications of collagen (eg. pentosidine, vesperlysine, GOLD, MOLD, CML) - Type I collagen C-telopeptide isomerization (α/β CTX ratio) -Posttranslational modifications of non-collagenous proteins (e.g. carboxylation and isomerization of osteocalcin)

TRACP 5b: tartrate-resistant acid phosphatase isoenzyme 5b; OPG: osteoprotegerin; RANK-L: receptor activator of nuclear factor kB ligand; Wnt: Wingless; Dkk-1: Dickkopf-1; sFRP: soluble Frizzled-related protein; GOLD (glyoxal-derived lysine dimer); MOLD (methylglyoxal-derived lysine dimer); CML (carboxymethyllysine)

Table 1: New candidate biochemical markers of bone metabolism by category.

Based on the available limited clinical data, measurements of serum BSP may be particularly interesting for the clinical investigation of patients with cancer. However, accurate measurements of serum BSP with currently available immunoassays remain challenging, especially because of its tight association with circulating factor H.

Other non-collagenous proteins, including *osteopontin*, which belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) family like BSP, and *periostin*, a secreted adhesion molecule with preferential distribution in the periosteum envelope, have also been suggested to provide information on bone metabolism, especially related to cancer-induced bone disease (16; 17). Clinical data from other metabolic bone diseases, including postmenopausal osteoporosis, are currently unavailable.

Although most of newly synthesized *osteocalcin* is captured by bone matrix, a small fraction is released into the blood where it can be detected by immunoassays and is currently considered as a specific bone formation marker. Circulating osteocalcin is constituted of different immunoreactive forms including the intact molecule, but also various fragments (18). The majority of these fragments is generated from the *in vivo* degradation of the intact molecule and thus also reflects bone formation (18). However, *in vitro* studies indicate that some osteocalcin fragments could also be released from osteoclastic degradation of bone matrix (19), resist glomerular filtration and accumulate in urine (20). Mass spectrometry analyses of urine from patients with Paget's disease and healthy children have shown that most of the urinary osteocalcin fragments consist of the mid-molecule portion (21), which appears to be protected from degradation in the kidney. Elevated levels of urinary osteocalcin were reported in osteoporotic postmenopausal women (20) and values decreased after one month of treatment with the bisphosphonate alendronate, in contrast to the absence of changes in serum total osteocalcin, suggesting that they reflect bone resorption. A recent study showed that urinary osteocalcin levels were associated with BMD loss at the spine and the hip as evaluated over 5 years in 601 postmenopausal women aged 75 years and older (22). In the same population, high levels of urinary osteocalcin – but not serum total osteocalcin – were associated with an increased risk of clinical vertebral fracture independent of BMD (23). Theoretically, urinary osteocalcin fragments may constitute a more specific bone resorption marker than type I collagen-related fragments, although their clinical value in osteoporosis remains to be more extensively evaluated.

Osteoclastic Enzymes

TRACP 5b

Acid phosphatase is a lysosomal enzyme that is present primarily in bone, prostate, platelets, erythrocytes and spleen. Bone acid phosphatase is resistant to L (+)-tartrate (TRACP), whereas the prostatic isoenzyme is inhibited. Acid phosphatase circulates in blood and shows higher activity in serum than in plasma because of the release of platelet phosphatase activity during the clotting process. In normal plasma, TRACP corresponds to plasma isoenzyme 5. Isoenzyme 5 is represented by two sub forms, 5a and 5b. TRACP 5a is derived mainly from macrophages and dendritic cells whereas TRACP 5b is more specific for osteoclasts (24;25). These two forms differ by their carbohydrate content, optimum pH and specific activity. TRACP 5a is a monomeric protein while TRACP 5b is cleaved in two subunits. In osteoclasts, TRACP 5b together with cathepsin K is localized in transcytotic vesicles that transport bone matrix degradation products from the resorption lacunae to the opposite functional secretory domain (26). *In vitro*, cathepsin K cleaves TRACP, resulting in its activation to generate reactive oxygen species that could then participate in finalizing matrix degradation during transcytosis.

Two different immunoassays for serum TRACP that preferentially detect the isoenzyme 5b have been developed more recently. The first one uses antibodies that recognize both intact and fragmented TRACP 5a and 5b, with selectivity for TRACP 5b being partly achieved by performing the measurements at optimal pH for TRACP 5b (27). More recently, a new immunoassay using two monoclonal antibodies raised against purified bone TRACP 5b that shows limited crossreactivity for TRACP 5a has been described (28). One antibody captures active intact TRACP 5b and the other eliminates interference of inactive fragments. We found that this new ELISA for TRACP 5b was more responsive to changes in bone turnover following menopause and alendronate therapy than the previous one (29).

Serum TRACP 5b is likely to represent mainly osteoclast number and activity and may thus provide complementary information on bone resorption compared to the type I collagen-related markers (30). For example, it has been shown that cathepsin K inhibitors given to postmenopausal women markedly decrease the serum and urine concentration of the type I collagen fragments CTX and NTX, while serum TRACP 5b remains unchanged, consistent with the mechanism of action of this class of drugs. Another advantage of serum TRACP 5b relates to its limited diurnal variation and negligible effect of food intake. This results in a lower intra-patient variability than type I collagen biochemical markers of bone resorption, although the magnitude of changes induced by anti-resorptive therapy such as bisphosphonates in postmenopausal women is also lower (31).

Cathepsin K

The enzyme cathepsin K is a member of the cysteine protease family that, unlike other cathepsins, has the unique ability to cleave both helical and telopeptide regions of collagen type I (32). The enzyme is produced as a 329 amino acid precursor procathepsin K, which is cleaved to its active form with a length of 215 amino acids, which *in vivo* is believed to occur in the low pH environment of the bone resorption lacunae. Commercially, two assays are available for measuring cathepsin K in serum that measure the enzymatic activity and the protein concentration, respectively, but clinical data are limited. Increased cathepsin K levels have been reported in patients with active rheumatoid arthritis (33;34), patients with Paget's disease (35) and postmenopausal women with fragility fractures (36), but not in patients with bone metastases from breast or prostate cancer (37). More recently, serum cathepsin K was shown to be associated with BMD loss at the femoral neck – but not lumbar spine – in 43 perimenopausal and early postmenopausal women followed for 3 years (38). Quite unexpectedly, in this study there was no significant association between serum cathepsin K and CTX, a type I collagen fragment released by this enzyme. Because the circulating concentration of

cathepsin K is very low and currently available assays lack sensitivity, accurate determination of the serum concentration of this enzyme remains challenging.

Regulators of Osteoclastic and Osteoblastic Activity

RANKL and OPG

The RANKL/RANK/OPG system is one of the main regulators of osteoclast formation and function (for a recent review, see (39)). The relevance of this pathway in postmenopausal bone loss has been demonstrated by Eghbali-Fatourechi *et al*. (40) who analyzed RANKL expression by bone marrow mononuclear cells and T and B lymphocytes from premenopausal women, untreated postmenopausal women and postmenopausal women treated with estrogen. They found that the levels of RANKL per cell were increased by two- to three-fold in untreated postmenopausal women, compared to premenopausal women, and correlated positively with serum and urine markers of bone resorption and negatively with circulating estradiol. There was, however, no difference between groups in circulating levels of soluble RANKL and OPG. More recently, it has been shown that the bisphosphonate risedronate reduces the differentiation of peripheral blood mononuclear cells into osteoclasts and their production of RANKL, although circulating levels again were not affected (41).

At present it remains unclear what proportion of circulating OPG is monomeric, dimeric or bound to RANKL and which of these forms is the most biologically relevant to measure. The same issues arise for the measurement of circulating soluble RANKL that, in its free form, is at barely detectable levels in healthy individuals. It is also possible that circulating levels of OPG and RANKL do adequately reflect local bone marrow production. A recent study analyzed the association between mRNA RANKL expression in the proximal femur and circulating levels of total soluble RANKL protein levels in 40 patients with osteoarthritis of the hip (42). In male subjects only and unexpectedly, there was a negative association between mRNA bone expression and circulating soluble levels for reasons that are unclear. These limitations probably explain the conflicting data available on the association of circulating OPG and RANKL (which could be positive, negative or nil) with BMD and biochemical markers of bone turnover in postmenopausal women and elderly men (43).

Wnt signaling molecules

The Wnt signaling pathway plays a pivotal role in the differentiation and activity of osteoblastic cells (44). There are 19 closely related Wnt genes that have been identified in humans. The primary receptors of Wnt molecules are the seven-transmembrane Frizzled-related proteins (FRPs), each of which interacts with a single transmembrane low-density lipid (LDL) receptor-related protein 5/6 (LRP5/6). Different secreted proteins, including soluble FRP-related proteins (sFRPs), Wnt inhibitory factor-1 (WIF1), and Dickkopfs (Dkk) 1 to 4 prevent ligand-receptor interactions and consequently inhibit the Wnt signaling pathway. Alterations of the Wnt signaling pathway and its regulatory molecules, including Dkk-1 and sFRP, have been shown to play an important role in bone turnover abnormalities associated with osteoporosis, arthritis, multiple myeloma, bone metastases from prostate and breast cancer, and arthritis (45). Immunoassays for circulating Dkk-1 have been developed recently. Increased circulating Dkk-1 levels have been reported in clinical situations characterized by markedly depressed bone formation as in multiple myeloma (46), or by increased focal osteolysis from multiple myeloma (46), and in bone metastases from breast (47) (Fig. 1) or lung cancer (48), and in rheumatoid arthritis (49). Conversely, in patients with osteoarthritis of the hip, a disease characterized by focal subchondral bone sclerosis, decreased serum Dkk-1 has been shown to be associated with a lower risk of joint destruction (50). The role of circulating Dkk-1 and other Wnt signaling molecules in the clinical investigation of postmenopausal osteoporosis remains to be determined.

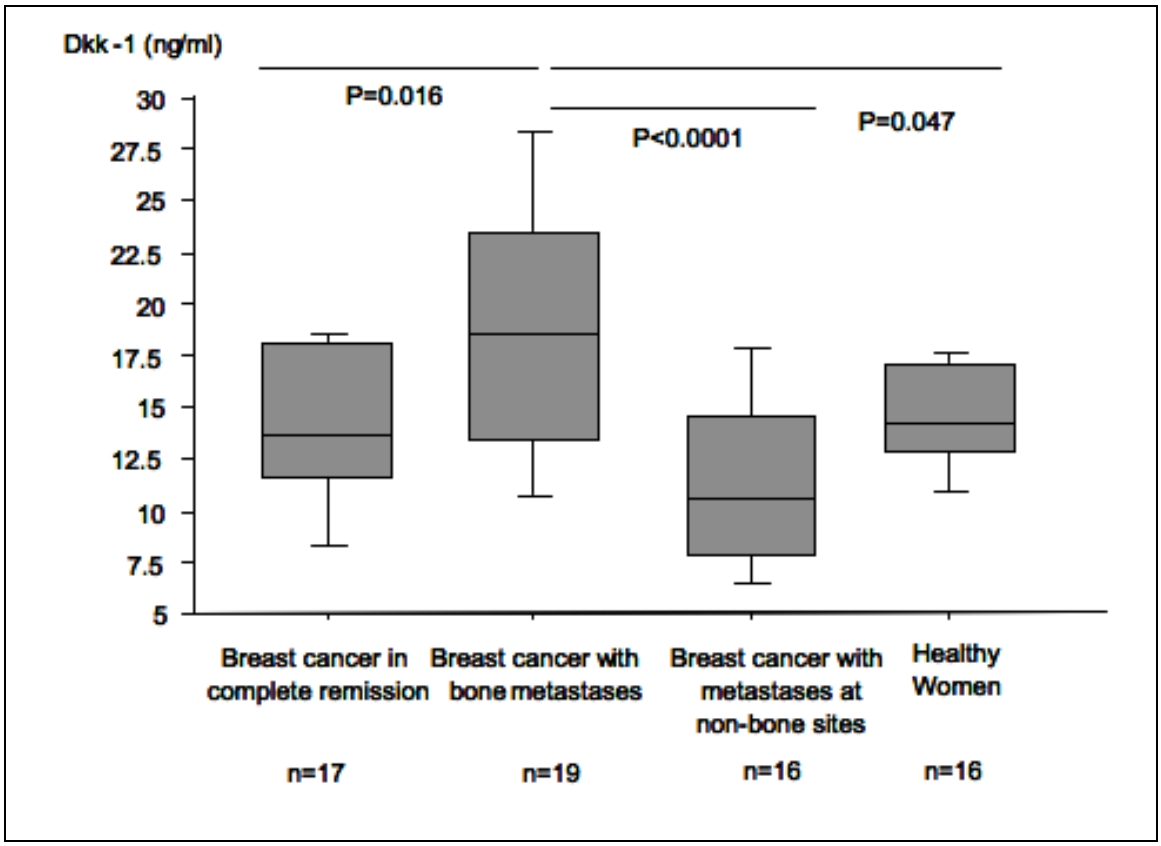

Fig. 1: Increased circulating levels of Dickkopf-1 (Dkk-1) in bone metastases from breast cancer. From the bottom up, the box indicates the 25th, 50th (median) and 75th percentiles, while the bars indicate the 10th and 90th percentiles, respectively. From Voorzanger-Rousselot N, Goehrig D, Journe F, Doriath V, Body JJ, Clézardin P, Garnero P. Increased Dickkopf-1 expression in breast cancer bone metastases. *Br J Cancer*. 2007 Oct 8;97(7):964-70. Used with permission.

Posttranslational Modifications of Bone Matrix Proteins

Collagen crosslinking and isomerization

In bone matrix, type I collagen molecules undergo a series of enzymatic and non-enzymatic intra- and extracellular posttranslational modifications (Fig. 2). These include the formation of enzymatic crosslinks within the N- and C-telopeptides that are initiated by the conversion of lysine and hydroxylysine residues through the activity of the lysyl oxidase, by the non-enzymatic Advanced Glycation End products (AGEs) that occur spontaneously in the presence of extracellular sugars and by the isomerization of the aspartic acid in the C-telopeptide (Fig. 2). Isomerization results in the conversion of the native sequence (α CTX) within the newly synthesized collagen molecule where the aspartic acid (D) is linked to the adjacent glycine (G) residue through its carboxyl-group in position α into a β-isomerized form (so-called β CTX) where the peptide bond now involved is the carboxyl group in position β of aspartic acid (51;52). In contrast to the enzymatic telopeptide crosslinks and some non-enzymatic AGEs (*e.g.*, pentosidine), this spontaneous non-enzymatic isomerization process does not lead *per se* to the formation of crosslinks between adjacent collagen molecules. However, isomerization does alter the conformation of the type I collagen C-telopeptide by introducing a kink in the peptide backbone (51;52).

The pattern of age-related changes of these various collagen post-translational modifications differs.

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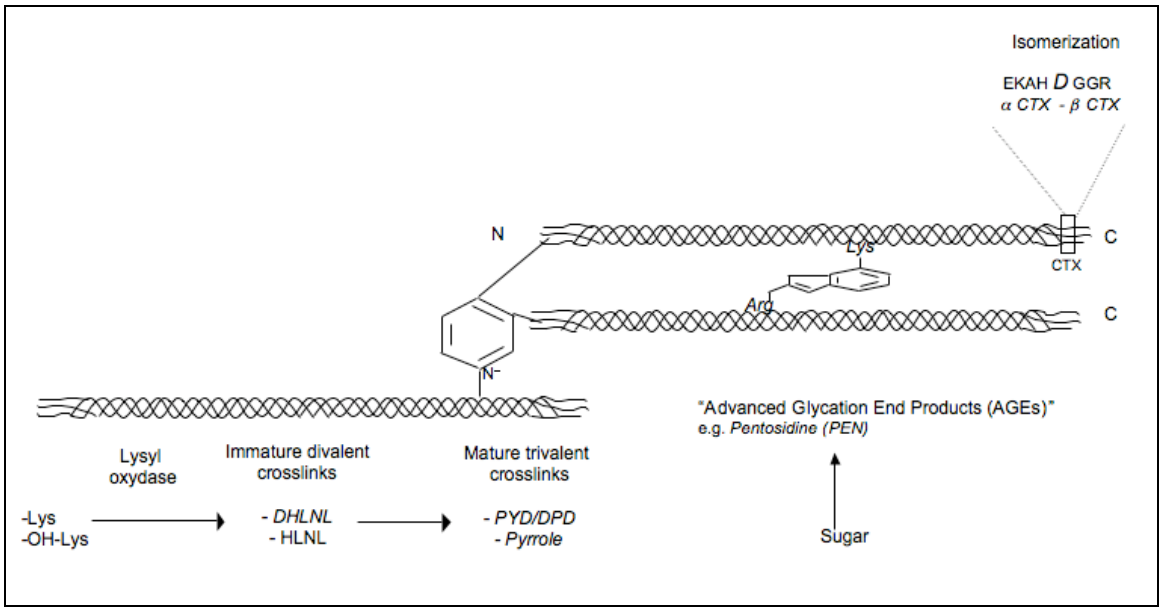

Fig. 2: Schematic representation of the extracellular post-translational modifications of type I collagen in bone matrix. Type I collagen is formed by the association of two α1 chains and one α2 chain in a triple helix, except at the two ends (N- and C-telopeptides). In bone matrix, type I collagen is subjected to different post-translational modifications: the mature trivalent crosslinks including pyridinoline (PYD), deoxypyridinoline (DPD) and pyrrole that make bridges between 2 telopeptides and the helicoidal region of another collagen molecule. These molecules result from the maturation of divalent crosslinking molecules (dihydroxylysinornorleucine, DHLNL and hydroxylysinonorleucine, HLNL) whose synthesis requires an enzymatic process (lysyl oxydase); the Advanced Glycation End products (AGEs) that are formed by non-enzymatic glycation when sugar (*e.g.*, glucose) is present in the extracellular matrix. Some AGEs, such as pentosine, are crosslinking molecules although their precise location remains to be determined; the non-enzymatic isomerization of aspartic acid (D) occurring in the C-telopeptides of α1 chains (see text for details).

Enzymatic telopeptide pyridinoline crosslinks increase up to about 30-40 years, but then plateau with skeletal maturity, whereas AGEs accumulate with age in human cortical bone (Fig. 2) (53). The kinetic of aspartic acid isomerization at 37°C has been studied *in vitro* using synthetic CTX peptides and immature fetal bovine bone collagen extract that consists mainly of α CTX isomer (52). At equilibrium of the reaction, about 20% of CTX peptide remains in its original α form and 80% is β-isomerized (52). Thus, in contrast to AGEs, the relative concentration of isomerized β CTX vs. α CTX cannot exceed the level reached at the equilibrium of the kinetic of isomerization. The ratio between native and isomerized CTX (α/β CTX) measured in urine by specific immunoassays gives an accurate estimate of the extent of type I collagen isomerization in bone tissue (52). Because of high bone remodeling in children, the equilibrium of the kinetic of isomerization cannot be achieved, resulting in a relatively higher proportion of α CTX compared to β CTX (high α/β CTX ratio) (Fig. 3). In adults, because on average the rate of bone remodeling is slower than the kinetic of isomerization, the equilibrium is achieved, resulting in a fairly constant α/β CTX ratio from the age of 20 and up (Fig. 3). As discussed below, the α/β CTX ratio can be altered in some postmenopausal women and with diseases characterized by high or low bone remodeling.

Several *ex vivo* and *in vivo* studies have shown that the extent of the collagen posttranslational modifications described above may contribute to bone fracture resistance including bone strength and toughness (54). Banse *et al*. (55) showed that the ratio between the enzymatic crosslinks pyridinoline (PYD)/deoxypyridinoline (DPD) was significantly associated with the

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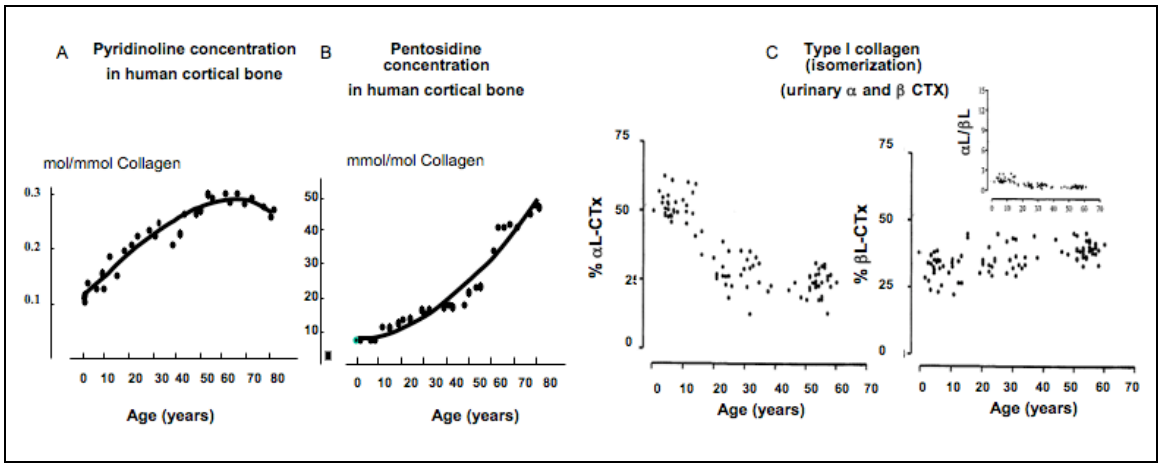

Fig. 3: Age-related changes of enzymatic crosslinks (A), glycation-mediated non-enzymatic crosslinks (B), and aspartic isomerization of C-telopeptide (C) in healthy adults. The enzymatic pyridinoline (A) and glycation-mediated non-enzymatic pentosidine (B) crosslinks were analyzed in human diaphysial femurs from 29 male subjects (From Saito M, Marumo K, Fujii K, Ishioka N. Single-column high-performance liquid chromatographic-fluorescence detection of immature, mature, and senescent cross-links of collagen. *Anal Biochem.* 1997 Nov 1;253(1):26-32). The relative content of native (α CTX) and isomerized (β CTX) type I collagen C-telopeptides was analyzed in urine samples from 114 healthy individuals by specific ELISA (C). The insert on the right panel shows the ratio of α/β CTX (From Cloos PA, Fledelius C. Collagen fragments in urine derived from bone resorption are highly racemized and isomerized: a biological clock of protein aging with clinical potential. *Biochem J*. 2000 Feb 1;345 Pt 3:473-80). Used with permission.

compressive biomechanical properties of human vertebrae independent of BMD. Preclinical *in vivo* studies and clinical studies relating homocysteinuria to fracture risk also support a role for enzymatic collagen crosslinks in bone fracture resistance. Homocysteinuria is a rare autosomal recessive disease that is characterized by generalized osteoporosis and increased bone fragility. Increased bone fragility has been attributed to the interference of homocysteine with the formation of enzymatic telopeptide collagen crosslinking (56). A recent rat study showed that increased homocysteinemia induced by a diet enriched in methiononine or homocysteine for 3 months resulted in decreased bone formation, bone volume and bone strength at the distal femur (57). Epidemiological studies investigating the association between plasma homocysteine levels and fracture risk have been inconsistent. Increased plasma homocysteine levels were found to be associated with increased fracture risk in elderly men and women (58-60), with stronger association in men (58), especially when combined with low vitamin B12 levels (60). However, such associations were not confirmed more recently in younger (mean age 62 years) (61) and older (> 75 years) (62) postmenopausal women after adjustment for age.

The role of the non-enzymatic AGEs in bone fracture resistance has also been supported by a series of *ex-vivo* cadaveric experiments and clinical studies. Wang *et al*. (63) showed that higher levels of pentosidine in human cortical bone were associated with decreased resistance to fracture and, more specifically, toughness. Similar findings were reported more recently in human vertebral bone tissue (64;65). High bone pentosidine content was also recently reported in patients with hip fractures (66). High AGE content in bone tissue is likely to result in abnormally high collagen crosslinking that would lead to decreased ductility, *i.e*., the ability of bone to deform and absorb energy upon mechanical loading. In 432 elderly Japanese women, increased urinary pentosidine was moderately associated with an increased risk of incident vertebral fracture independent of BMD and the systemic levels of bone turnover markers (67). A clinical situation where AGEs may be particularly relevant is type 2 diabetes, a

disease characterized by altered glucose metabolism and increased bone fragility despite increased BMD and low bone turnover (68). A recent study has shown an association between increased serum pentosidine levels and the presence of prevalent vertebral fracture in postmenopausal women with type 2 diabetes – but not in men – independent of several confounding factors including levels of glycated albumin, BMD and renal function (69) (Table 2). Pentosidine is only one of the several AGEs present in bone matrix. Other AGEs have been shown to accumulate in bone tissue including vesperlysine, methylglyoxal-derived lysine dimer (MOLD), glyoxal-derived lysine dimer (GOLD), imidazolone and N(epsilon)-carboxymethyllysine (70), although their association with bone fracture resistance has not yet been investigated. An important limitation of the currently identified AGEs is that they are not specific to bone and accumulate in various tissues including cartilage and skin with aging. Thus systemic measurements of currently identified AGEs only poorly correlated with bone tissue content (71). The identification of the major AGEs specific to bone tissue that directly affect mechanical properties would be extremely useful in developing biological markers reflecting changes of bone matrix maturation.

Gender	Odds-ratio* of prevalent fracture per 1 SD increase in serum pentosidine	
	Odds-ratio (95% CI)	P value
Female	0.79 (0.41-1.52)	0.4746
Male	2.50 (1.09-5.73)	0.0302

*Serum pentosidine levels were adjusted for age, body weight and height, glycated albumin, glomerular filtration rate, duration of diabetes, duration of postmenopausal status for women, presence of diabetic retinopathy or neuropathy, history of use of insulin or pioglitazone, smoking and alcohol intake, history of nonvertebral fracture and BMD.

Table 2: Increased serum pentosidine and the risk of prevalent vertebral fracture in type 2 diabetes. The study included 77 male (28 with prevalent vertebral fracture) and 76 female (20 with prevalent vertebral fractures) patients with type 2 diabetes. From Yamamoto M, Yamaguchi T, Yamauchi M, Yano S, Sugimoto T. Serum pentosidine levels are positively associated with the presence of vertebral fractures in postmenopausal women with type 2 diabetes. *J Clin Endocrinol Metab*. 2007 Dec 26; [Epub ahead of print]. Used with permission.

The role of isomerization of type I collagen in bone fracture risk has also been recently investigated. Histological studies have shown that in clinical situations characterized by a marked increase of bone remodeling in localized areas of the skeleton, such as in patients with Paget's disease (72) and bone metastases (73), the equilibrium of the kinetic of type I collagen isomerization cannot be achieved, resulting in a relatively higher proportion of α vs β CTX. Interestingly, both in patients with Paget's disease and bone metastases from breast cancer, this biochemical alteration of type I collagen was shown to be associated with the presence of woven bone – a tissue characterized by disorganized collagen fibers and increased fragility. In patients with Paget's disease of bone, this alteration of collagen maturation is reflected by a marked elevation of urinary α/β CTX ratio (72). Urinary α/β CTX ratio normalizes after treatment with bisphosphonates (74;75), a treatment that has been shown to result in the formation of a bone matrix with normal lamellar structure. Another clinical situation characterized by disorganized collagen matrix and increased bone fragility despite normal BMD in some individuals is osteogenesis imperfect (OI), a genetic disorder caused by mutations in the

type I collagen genes. In adults with OI, we recently found an increased urinary α/β CTX ratio (76), although to a lower magnitude than in Paget's disease of bone.

The investigation of type I collagen isomerization may also be of clinical relevance in postmenopausal osteoporosis. In the OFELY prospective study, we found that women with a urinary α/β CTX ratio in the highest quartile had an increased risk of incident fracture independent of both the level of hip BMD and of bone turnover rate measured by serum bone ALP (77) (Table 3). Conversely, when the urinary α/β CTX ratio was too low, *i.e.*, in the lowest quartile of the population, Byrjalsen *et al*. reported an increased risk of fracture in postmenopausal women (78). Thus, it is possible that the association between maturation of collagen as reflected by AGE content or type I collagen C-telopeptide isomerization and bone fragility follows a U-shaped pattern (Fig. 4), with maturation and crosslinking that is too high or too low compromising tissue strength. Interestingly, *in vitro* maturation of fetal bovine cortical bone, characterized initially by very low collagen maturation (*e.g.*, low enzymatic crosslinks, low CTX isomerization and very low AGE content) to an extent observed in adult animals results in improved bending and compressive mechanical properties (79).

Urinary CTX ratio at baseline	Relative Risk* (95% CI) of fracture for values in the upper quartile	
	All Fractures	Non-vertebral fractures only
α/β CTX		
Unadjusted	2.0 (1.2-3.5)	2.5 (1.3-4.6)
Adjusted for bone ALP	1.8 (1.1-3.2)	2.2 (1.1-4.2)
Adjusted for femoral neck BMD	1.8 (1.03-3.1)	2.2 (1.2-4.0)
Adjusted for bone ALP + femoral neck BMD	1.7 (0.95-2.9)	2.0 (1.04-3.8)

* Adjusted for age, presence of prevalent fracture, and physical activity

Table 3: Increased urinary α/β CTX ratio as an independent predictor of the risk of osteoporotic fractures. Four hundred and eight women participating in the OFELY study were followed prospectively for 6.8 years. 55 non-vertebral fractures and 16 incident vertebral fractures were recorded. The table shows the relative risks of fracture for women with baseline levels of α/β CTX in the upper quartile. From Garnero P, Cloos P, Sornay-Rendu E, Qvist P, Delmas PD. Type I collagen racemization and isomerization and the risk of fracture in postmenopausal women: the OFELY prospective study. *J Bone Miner Res*. 2002 May;17(5):826-33. Used with permission.

The fracture efficacy of anti-resorptive therapy is only partly explained by changes in BMD and bone turnover (80), suggesting that other factors, including modifications of bone matrix properties, play a role. The effects of anti-resorptive therapies on the non-enzymatic modifications of collagen have been investigated in *ex vivo* animal studies and *in vivo* clinical studies. In vertebral trabecular bone from dogs, treatment with the bisphosphonate alendronate and risedronate – but not raloxifene – induced a decrease of the α/β CTX ratio and a marked increase of pentosidine, suggesting increased bone collagen maturation with bisphosphonates (81). These animal data are consistent with the decrease of the urinary α/β CTX ratio observed in postmenopausal women receiving alendronate at 10 or 20 mg/day and oral daily (2.5 mg) or intermittent ibandronate, whereas no significant change was observed with raloxifene or estradiol (82) (Fig. 5). However, in another study performed in postmenopausal women with osteoporosis participating in the PaTH study, we could not find a significant change

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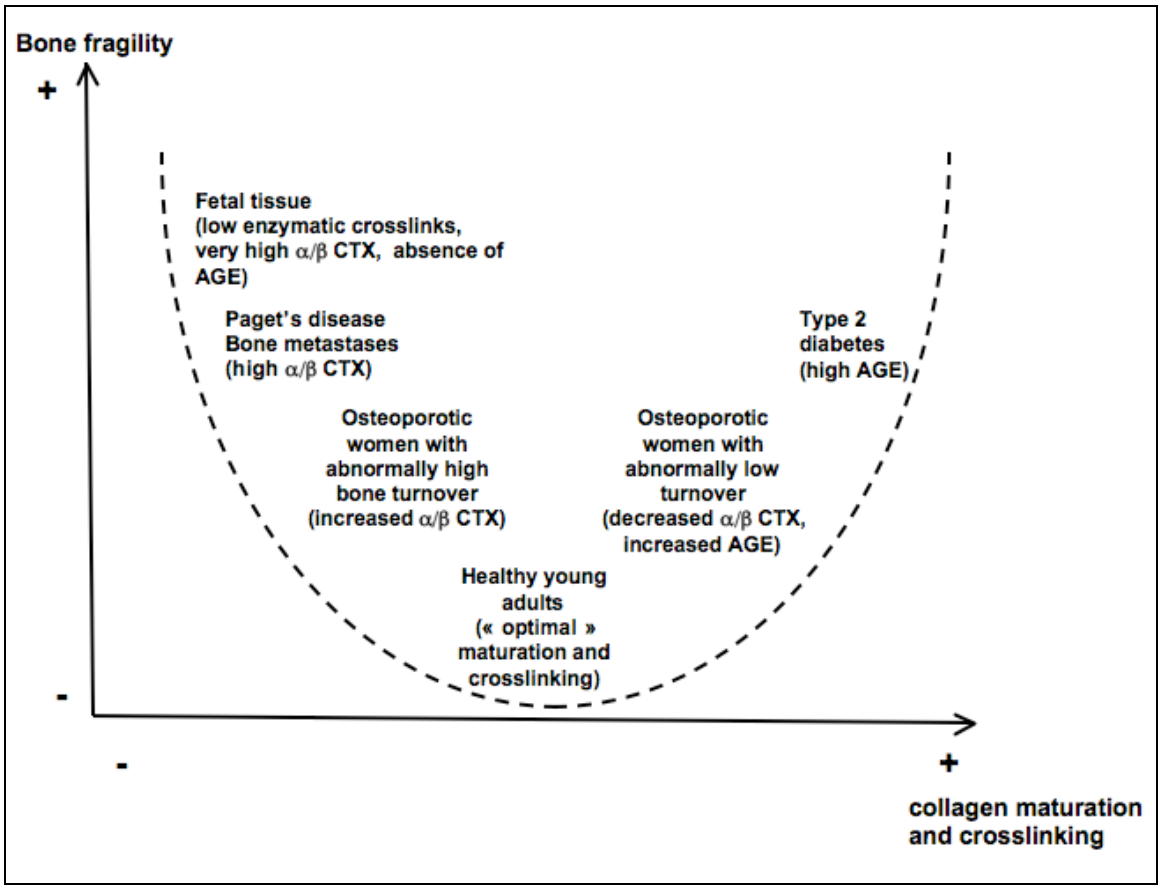

Fig. 4: Hypothetical relationship between collagen maturation and bone fragility with age and disease. The α/β CTX ratio reflects isomerization of aspartic acid within the C-telopeptide of the type I collagen α1 chain, with a high ratio indicative of a low degree of isomerization. AGE: non-enzymatic Advanced Glycation End products.

in the α/β CTX ratio after 1 or 2 years with the lower dose of 10 mg/day of alendronate (83). This suggests that a profound suppression of bone turnover may be required to induce detectable changes in the urinary α/β CTX ratio. Whether these changes in collagen maturation observed with bisphosphonate therapy will translate into alterations of fracture resistance independent of BMD remains to be investigated.

Altogether, these data indicate that the degree of posttranslational modifications – more specifically non-enzymatic age-related-modifications of collagen – plays an independent role in determining the bone mechanical competence and that the ratio of α/β CTX may provide an *in vivo* marker of bone matrix maturation.

Non-collagenous proteins

Bone matrix also contains non-collagenous proteins that can undergo post-translational modifications. Osteocalcin contains three residues of γ-carboxyglutamic acid (GLA). GLA results from the carboxylation of glutamic acid residues, an intracellular posttranslational modification that is vitamin K-dependent. In elderly women, levels of undercarboxylated osteocalcin – which can be evaluated indirectly by the method of incubation of serum with hydroxyapatite or directly by ELISA – above the premenopausal range were associated with a two- to three-fold increase in the risk of hip fracture, although total osteocalcin was not predictive (84;85). The mechanisms relating increased undercarboxylation of osteocalcin and fracture risk is unclear. Serum undercarboxylated osteocalcin (86), but not

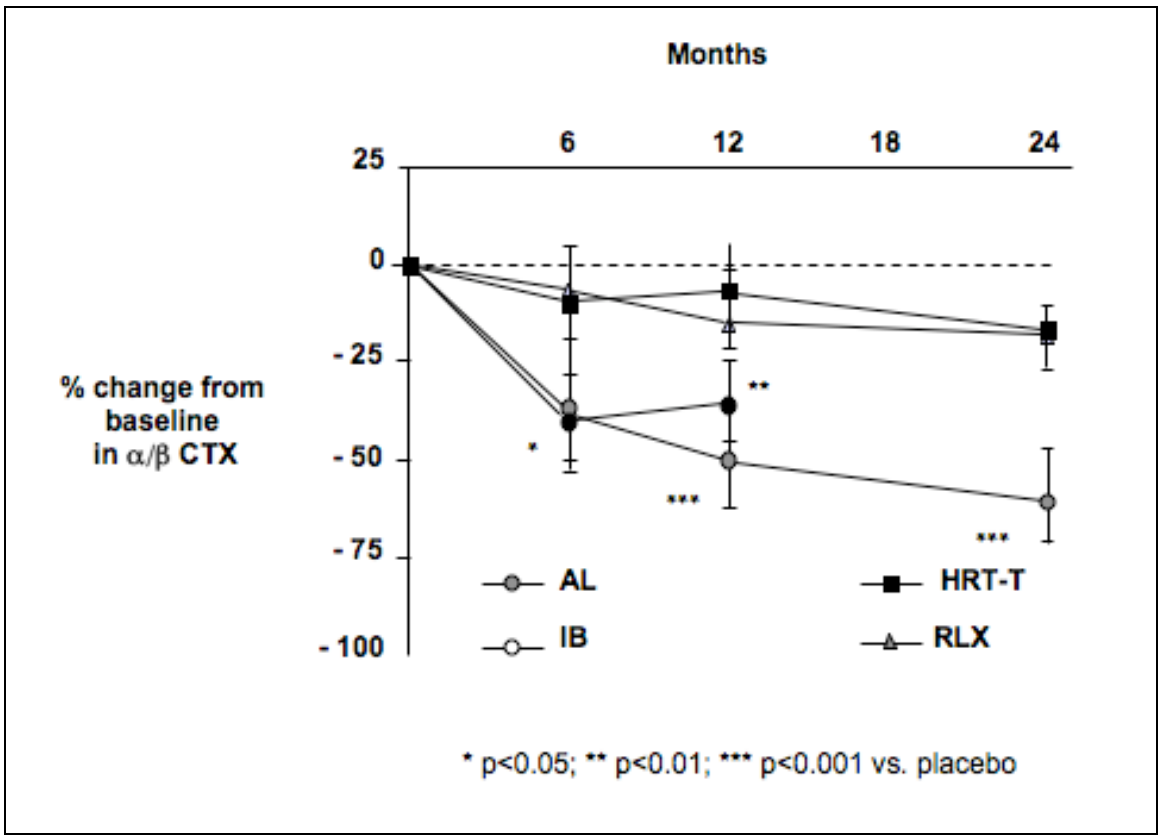

Fig. 5: Effects of different anti-resorptive treatments on type I collagen isomerization in postmenopausal women. The graph shows the mean (SEM) changes from baseline in the urinary α/β CTX ratio in postmenopausal women receiving the bisphosphonates alendronate [AL; 10 mg/day (n=14) or 20 mg/day (n=13)], ibandronate [IB; 2.5 mg/day (n=36), intermittent 20 mg every 2nd day for 24 days every 3 months (n=36)], transdermal estradiol [HRT-T; 45 μg 17 β estradiol/day combined with 40 μg levonorgestrel, (n=35)] or raloxifene [RLX, 60 mg/day; (n=30)]. Because these were not head-to-head comparison trials, for each treatment group the changes from baseline were adjusted for the changes in the corresponding placebo groups. From Byrjalsen I, Leeming DJ, Qvist P, Christiansen C, Karsdal MA. Bone turnover and bone collagen maturation in osteoporosis: effects of antiresorptive therapies. *Osteoporos Int*. 2008 Mar;19(3):339-48. Used with permission.

total osteocalcin, has been found to be associated more strongly with ultrasonic transmitted velocity (which has been suggested to reflect in part changes in bone microarchitecture) at the os calcis and tibia than with BMD. Osteocalcin also contains in its sequence 5 residues of aspartic acid that can undergo isomerization like type I collagen. Although not directly analyzed in bone matrix, isomerized osteocalcin fragments have been described in patients with Paget's disease of bone (87). The influence of the isomerization and other posttranslational modifications of non-collagenous proteins on the mechanical competence of bone matrix remain to be investigated.

Conclusion

New biochemical markers reflecting different biological pathways of osteoblastic and osteoclastic activity and changes in structural properties of bone matrix have been developed. Further studies are required to delineate their respective clinical value alone and in combination with existing markers in osteoporosis. With the optimization of proteomic-based technologies the list of available markers should further expand in the near future (88).

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