

Review Article

Biochemical Markers of Bone Turnover

Part I : Biochemistry and Variability

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Abstract

With the ageing population in most countries, disorders of bone and mineral metabolism are becoming increasingly relevant to every day clinical practice. Consequently, the interest in, and the need for effective measures to be used in the screening, diagnosis and follow-up of such pathologies has markedly grown. Together with clinical and imaging techniques, biochemical tests play an important role in the assessment and differential diagnosis of metabolic bone disease.

In recent years, the isolation and characterisation of cellular and extracellular components of the skeletal matrix have resulted in the development of molecular markers that are considered to reflect either bone formation or bone resorption. These biochemical indices are non-invasive, comparatively inexpensive and, when applied and interpreted correctly, helpful tools in the diagnostic and therapeutic assessment of metabolic bone disease.

Part I of this article provides an overview of the basic biochemistry of bone markers, and sources of non-specific variability. Part II (to be published in a subsequent issue of this journal) will review the current evidence regarding the clinical use of biochemical markers of bone remodelling in metabolic and metastatic bone disease.

Introduction

Bone is a metabolically active tissue that undergoes continuous remodelling by two counteracting processes, namely bone formation and bone resorption. These processes rely on the activity of osteoclasts (resorption), osteoblasts (formation) and osteocytes (maintenance). Under normal conditions, bone resorption and formation are tightly coupled to each other, so that the amount of bone removed is always equal to the amount of newly formed bone (Figure 1). This balance is achieved and regulated through the action of various systemic hormones (e.g. PTH, vitamin D, other steroid hormones) and local mediators (e.g. cytokines, growth factors). In contrast, somatic growth, ageing, metabolic bone diseases, states of increased or decreased mobility, therapeutic interventions and many other conditions are characterised by more or less pronounced imbalances in bone turnover. The results of such uncoupling in bone turnover are often changes in bone structure, strength and mass. While bone structure and strength are difficult to measure *in vivo*, bone mass can be assessed by densitometric techniques. In contrast to these rather static measures, however, molecular markers of bone

metabolism are helpful tools to detect the dynamics of the metabolic imbalance itself.^{1,2}

Although the currently available markers of bone turnover include both enzymes and non-enzymatic peptides derived from cellular and non-cellular compartments of bone, they are usually classified according to the metabolic process they are considered to reflect. Most biochemical indices of bone resorption are related to collagen breakdown products such as hydroxyproline or the various collagen cross-links and telopeptides. Other markers of bone resorption include non-collagenous matrix proteins such as bone sialoprotein (BSP), or osteoclast-specific enzymes like tartrate-resistant acid phosphatase or cathepsin K. In contrast, markers of bone formation are either by-products of collagen neosynthesis (e.g. propeptides of type I collagen), or osteoblast-related proteins such as osteocalcin (OC) and alkaline phosphatase (AP). For clinical purposes, therefore, markers of bone formation are distinguished from indices of bone resorption (Table 1, Figure 2). This distinction, however, is not as sharp as it may appear. For example, some marker components reflect, at

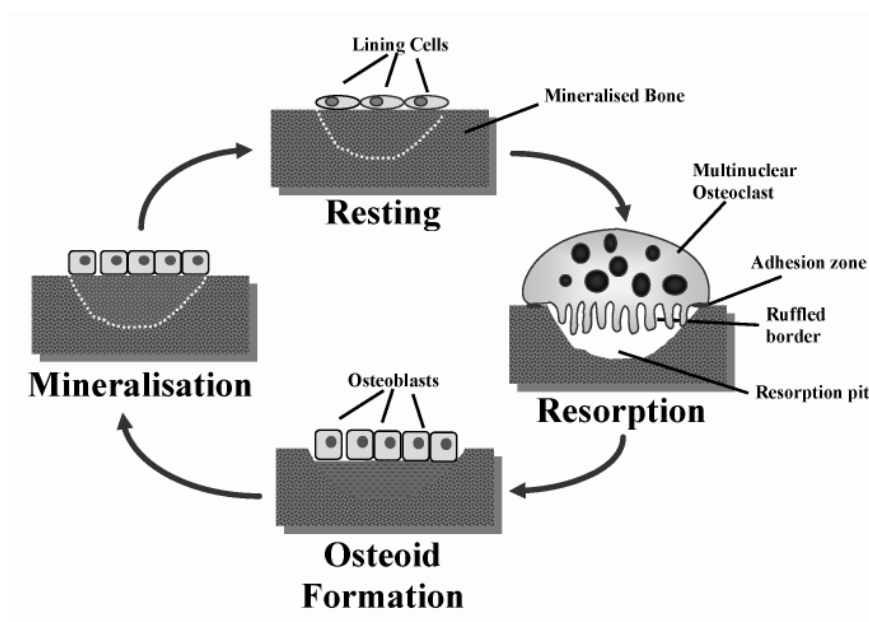


Figure 1. The bone remodelling cycle. Under normal conditions, the resorption (osteoclast) phase takes approximately 10 days, which is then followed by a formation (osteoblast) phase that can last for up to 3 months.

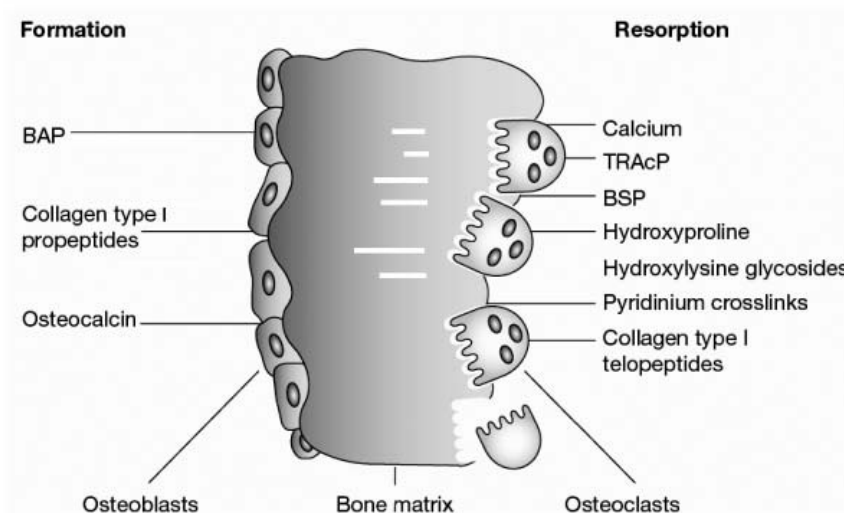


Figure 2. Biochemical markers of bone remodelling.

least in part, both bone formation and bone resorption (e.g. hydroxyproline, certain OC fragments). Furthermore, most of the molecules used as markers of bone turnover are also present in tissues other than bone, and non-skeletal processes

may therefore influence their circulating or urinary levels. Finally, changes in markers of bone turnover are not disease specific but reflect, as an integral measure, alterations in the metabolism of the entire skeletal envelope independently

Table 1. Markers of bone turnover.

Marker	Tissue of Origin	Specimen	Analytical Method	Remarks
Markers of bone formation				
Bone-specific alkaline phosphatase (BAP, bone ALP)	Bone	Serum	Electrophoresis, Precipitation, IRMA, EIA	Specific product of osteoblasts. Some assays show up to 20% cross-reactivity with the liver isoenzyme (LAP)
Osteocalcin (OC)	Bone, platelets	Serum	RIA, IRMA, ELISA	Specific product of osteoblasts; many immunoreactive forms in blood; some may be derived from bone resorption.
C-terminal propeptide of type I procollagen (PICP)	Bone, soft tissue, skin	Serum	RIA, ELISA	Specific product of proliferating osteoblasts and fibroblasts.
N-terminal propeptide of type I procollagen (PINP)	Bone, soft tissue, skin	Serum	RIA, ELISA	Specific product of proliferating osteoblast and fibroblasts; partly incorporated into bone extracellular matrix.
Markers of bone resorption				
Collagen-related markers				
Hydroxyproline, total and dialysable (Hyp)	Bone, cartilage, soft tissue, skin	Urine	Colorimetry HPLC	Present in all fibrillar collagens and partly collagenous proteins, including C1q and elastin. Present in newly synthesised and mature collagen, i.e. both collagen synthesis and tissue breakdown contribute to urinary hydroxyproline.
Hydroxylysine-glycosides	Bone, soft tissue, skin, serum complement	Urine (serum)	HPLC ELISA	Hydroxylysine in collagen is glycosylated to varying degrees, depending on tissue type. Glycosylgalactosyl-OHLys in high proportion in collagens of soft tissues, and C1q; Galactosyl-OHLys in high proportion in skeletal collagens.

Pyridinoline (PYD)	Bone, cartilage, tendon, blood vessels	Urine Serum	HPLC ELISA	Collagens, with highest concentrations in cartilage and bone; absent from skin; present in mature collagen only.
Deoxypyridinoline (DPD)	Bone, Dentin	Urine Serum	HPLC ELISA	Collagens, with highest concentration in bone; absent from cartilage or skin; present in mature collagen only.
Carboxyterminal cross-linked telopeptide of type I collagen (ICTP, CTX-MMP)	Bone, Skin	Serum	RIA	Collagen type I, with highest contribution probably from bone; may be derived from newly synthesised collagen.
Carboxyterminal cross-linked telopeptide of type I collagen (CTX-I)	All tissues containing type I collagen	Urine (α-/ β) Serum (β only)	ELISA RIA	Collagen type I, with highest contribution probably from bone. Isomerisation of aspartyl to β-aspartyl occurs with ageing of collagen molecule.
Aminoterminal cross-linked telopeptide of type I collagen (NTX-I)	All tissues containing type I collagen	Urine Serum	ELISA CLIA RIA	Collagen type I, with highest contribution from bone.
Collagen I alpha 1 helicoidal peptide (HELP)	All tissues containing type I collagen	Urine	ELISA	Degradation fragment derived from the helical part of type I collagen (alpha-1 chain, AA 620-633). Correlates highly with other markers of collagen degradation, no specific advantage or difference in regards to clinical outcomes.

Non-Collagenous Proteins

Bone Sialoprotein (BSP)	Bone, Dentin, hypertrophic cartilage	Serum	RIA ELISA	Acidic, phosphorylated glycoprotein, synthesised by osteoblasts and osteoclastic-like cells, laid down in bone extracellular matrix. Appears to be associated with osteoclast function.
Osteocalcin fragments (ufOC, U-Mid-OC, U-LongOC)	Bone	Urine	ELISA	Certain age-modified OC fragments are released during osteoclastic bone resorption and may be considered an index of bone resorption.

Osteoclast Enzymes

Tartrate-resistant acid phosphatase (TRAcP)	Bone Blood	Plasma Serum	Colorimetry RIA ELISA	Six isoenzymes found in human tissues (osteoclasts, platelets, erythrocytes). Band 5b predominant in bone (osteoclasts).
Cathepsins (e.g. K, L)	K: Primarily in osteoclasts L: Macrophage, Osteoclasts	Plasma, Serum	ELISA	Cathepsin K, a cysteine protease, plays an essential role in osteoclast-mediated bone matrix degradation by cleaving helical and telopeptide regions of collagen type I. Cathepsin K and L cleave the loop domain of TRAP and activate the latent enzyme. Cathepsin L has a similar function in macrophages. Tests for measurement of Cathepsins in blood are presently under evaluation.

of the underlying cause. Hence, results of bone marker measurements should always be interpreted against the background of their basic science and the clinical picture.

Markers of Bone Formation

Bone formation markers are products of active osteoblasts expressed during different phases of osteoblast development. They are considered to reflect different aspects of osteoblast function and of bone formation. All markers of bone formation are measured in serum or plasma.

Serum Total Alkaline Phosphatase (AP)

AP is a ubiquitous, membrane-bound tetrameric enzyme attached to glycosyl-phosphatidylinositol moieties located on the outer cell surface.³ The precise function of the enzyme is yet unknown, but it obviously plays an important role in osteoid formation and mineralisation.⁴ The total AP serum pool (TAP) consists of several dimeric isoforms, which originate from various tissues: liver, bone, intestine, spleen, kidney, and placenta. In addition, certain tumours may express macromolecular forms of AP (e.g. “Nagao AP”).^{5,6}

The physiological isoforms of AP are coded by four gene loci, including three tissue-specific and one non-tissue-specific gene on chromosome 1. The latter encodes for the most abundant isoforms, namely bone, liver, and kidney AP. The differences between these non-specifically encoded isoenzymes are solely due to post-translational modifications in the carbohydrate moiety.⁷ In adults with normal liver function, approximately 50% of the total AP activity in serum is derived from the liver, whereas 50% arises from bone.⁸ In children and adolescents the bone-specific isoenzyme predominates (up to 90%) because of skeletal growth.^{9,10}

Many techniques have been developed to differentiate between the two main isoforms of circulating AP, including heat denaturation, electrophoresis, precipitation, selective inhibition and, more recently, immunoassays.¹¹⁻¹⁵ In healthy adults, most methods show a good correlation between bone specific and total AP (Figure 3). The newer immunoassays allow simple and rapid quantitation of either enzyme activity or enzyme mass. However, like all of the other techniques, even these assays show a certain degree of cross-reactivity

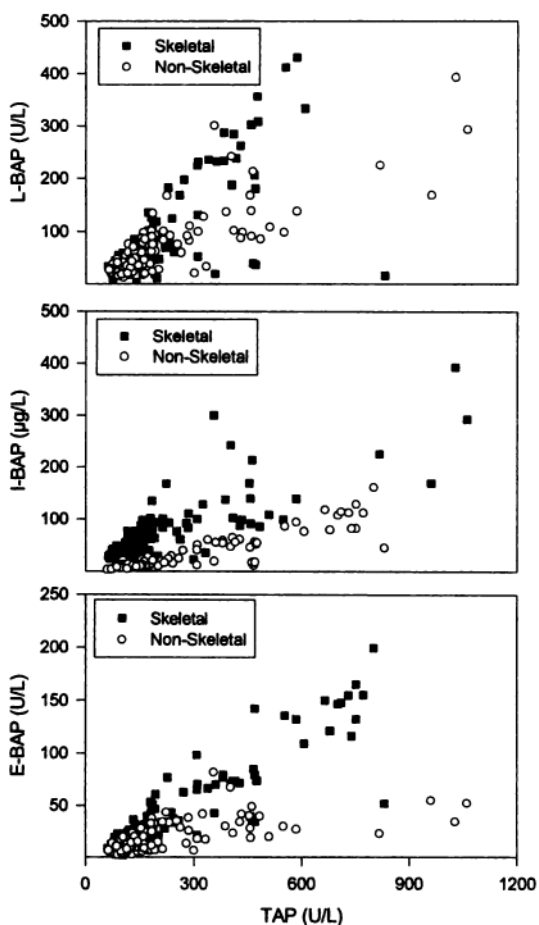


Figure 3. Correlation between serum total and bone specific alkaline phosphatase. Patients with non-skeletal disease had chronic hepatic failure, chronic obstructive pulmonary disease, or chronic renal failure. Patients with skeletal disease had Paget's disease of bone, primary or secondary hyperparathyroidism, or metastatic bone disease. TAP, total alkaline phosphatase; L-BAP, bone alkaline phosphatase measured by lectin precipitation assay; I-BAP, bone alkaline phosphatase measured by immunoradiometric assay (IRMA); E-BAP, bone alkaline phosphatase measured by enzyme linked immunosorbant assay (ELISA) Reproduced from, Woitge H, Seibel MJ, Ziegler R. Comparison of total and bone-specific alkaline phosphatase in patients with nonskeletal disorders or metabolic bone disease Clin Chem 1996;42:1796-1804 with permission of the publisher of Clinical Chemistry.

between bone and liver AP (15-20%). Therefore, in subjects with high liver AP, results of bone AP measurements may be artificially high, leading to false positive results.^{7, 16, 17}

Serum total AP is the most widely used marker of bone metabolism due to the wide availability of inexpensive and simple methods. Once liver disease is ruled out, serum levels

of total AP provide a good impression of the extent of new bone formation and osteoblast activity.^{18,19} From a clinical perspective, however, detection of the bone-specific AP (BAP) isoenzyme is increasingly preferred because of its higher specificity.^{8, 20, 21}

Osteocalcin (OC)

OC is a 5.8 kDa, hydroxyapatite-binding, protein exclusively synthesised by osteoblast, odontoblasts and hypertrophic chondrocytes.²²⁻²⁴ One of the major characteristics of OC is 3 vitamin-K dependent, gamma-carboxyglutamic acid (Gla) residues, which are responsible for the calcium binding properties of the protein.²⁵ At its carboxy-terminus, OC can also interact with other proteins, including cell surface receptors. These functions predispose OC as a molecule active in the organisation of the extracellular matrix. Earlier research has suggested that OC is involved in the process of osteoid mineralisation, as the protein is expressed mainly during this phase of bone formation. However, although OC has been known for more than 20 years, its precise function has yet to be determined. More recently, new light has been cast on this issue through the development of an OC knockout mouse model. Unexpectedly, these animals have increased cortical and trabecular thickness, and their bones seem mechanically more stable than those of the wild type.²⁶ Although the knockout model awaits further characterisation, it seems that OC is involved in the bone remodelling process and may act via a negative feed back mechanism.

OC is considered a specific marker of osteoblast function.²⁷ It is estimated that, directly after its release from osteoblasts, the largest part of the newly synthesised protein is incorporated into the extracellular bone matrix where it constitutes approximately 15% of the non-collagenous protein fraction. A smaller fraction is released into the circulation where it can be detected by immunoassays.²⁸⁻³⁴ Serum levels of immunoreactive OC have been shown to correlate well with the bone formation rate as assessed by histomorphometry.³⁵ However, the peptide is readily subject to rapid degradation in serum, so that both intact peptides and OC fragments of various sizes coexist in the circulation.³⁶⁻³⁸ Furthermore, since OC is incorporated into the bone matrix, some investigators have suggested that OC fragments may be released even during bone resorption. This may be particularly true for some smaller N-terminal fragments of OC, which are found in individuals with high bone turnover.³⁹⁻⁴¹ The ensuing heterogeneity of OC fragments in serum results in considerable limitations in the clinical application of this *a priori* highly specific marker. Thus, the various assays used to measure OC in serum detect fragments of various sizes and usually, epitope specificity and antibody cross-reactivity of the assays are ill defined. In practice, different immunoassays

have routinely yielded such varying results in measurements that they are not comparable.^{43,45}

Two-site immunoassays, utilising antibodies detecting different parts of the OC molecule, have been introduced that detect the intact 1-49 OC molecule. However, only one third of the total OC serum pool represents intact OC, and due to the instability of OC in serum, rapid loss of immunoreactivity is seen with these assays when samples are left for more than 1 hour at room temperature. To circumvent this problem, newer assays measure the largest degradation product of OC, the 1-43 (N-terminal / mid-molecule) fragment. This fragment, which represents one-third of the circulating OC pool, is a result of proteolytic degradation of the intact molecule and may in part be generated by active osteoblasts. Although little is known about the function of the N-terminal fragment, its measurement eliminates in part the problem of pre-analytical instability.^{45,46} However, quick processing of the blood sample after drawing is essential for most assays since a loss of reactivity is noted within a few hours at room temperature. The same applies to sera subjected to multiple thawing, or prolonged storage at temperatures above -25° C.

Procollagen Type I Propeptides

The procollagen type I propeptides are derived from collagen type I, the most abundant form of collagen found in bone.⁴⁵ However, type I collagen is also present in other tissues such as skin, dentin, cornea, vessels, fibrocartilage, and tendons. In bone, collagen is synthesised by osteoblasts in the form of pre-procollagen. These precursor molecules are characterised by short terminal extension-peptides: the amino (N-) terminal propeptide (PINP) and the carboxy (C-) terminal propeptide (PICP) (Figure 4).⁴⁶ After secretion into the extracellular space, the globular trimeric propeptides are enzymatically cleaved and liberated into the circulation.⁴⁷ PICP has a MW of 115 kDa, is stabilised by disulphide bonds, cleared by liver endothelial cells via the mannose receptor and therefore has a short serum half-life of 6-8 minutes.^{48,49} PINP has a MW of only 70 kDa, is rich in proline and hydroxyproline and is eliminated from the circulation by liver endothelial cells by the scavenger receptor. Since both PICP and PINP are generated from newly synthesised collagen in a stoichiometric fashion, the propeptides are considered quantitative measures of newly formed type I collagen. Although type I collagen propeptides may also arise from other sources, most of the non-skeletal tissues exhibit a slower turnover than bone, and contribute very little to the circulating propeptide pool.

Both propeptides are currently measured by specific immunoassays.^{50,51} Different studies have shown good correlations between serum PICP levels and the rate of bone formation.^{52,53} While the clinical relevance of PICP in the

evaluation of metabolic bone diseases is still viewed with skepticism,^{54,55} serum PINP appears to be of greater diagnostic validity. From a practical point of view, the thermostability of the propeptides is an advantage in that extended transport and storage times are well tolerated without significant loss of signal. The propeptides share these properties with most of the parameters of collagen metabolism (e.g., crosslinks, ICTP, NTx, CTx, hydroxyproline; *vide infra*).

Markers of Bone Resorption

Except for tartrate-resistant acid phosphatase the majority of bone resorption markers are degradation products of bone collagen (Figure 5). More recently, non-collagenous proteins such as BSP, and osteoclast-derived enzymes such as cathepsin K and L have been investigated as markers of bone turnover.

Hydroxyproline (OHP)

OHP is formed intracellularly from the post-translational hydroxylation of proline and constitutes 12-14% of the total amino acid content of mature collagen. Ninety percent of the OHP liberated during the degradation of bone collagen is primarily metabolised in the liver.⁵⁶ Subsequently, it is excreted in the urine where it may be detected either as free or peptide-bound hydroxyproline by colorimetric or HPLC methods.^{57,58} Urinary OHP is usually considered to reflect bone resorption. However, it should be noted that significant amounts of urinary OHP are derived from the degradation of newly synthesised collagen.⁵⁹ In addition, hydroxyproline can be found in other tissues such as the skin and, moreover, liberated from the metabolism of elastin and C1q.^{60,61} Urinary hydroxyproline is therefore considered an unspecific index of collagen turnover and, consequently, has been largely replaced by more specific techniques.

Hydroxylysine-Glycosides

The hydroxylysine-glycosides arise during the post-translational phase of collagen synthesis and occur in two forms, glycosyl-galactosyl-hydroxylysine (GGHL) and galactosyl-hydroxylysine (GHL) (Figure 5).⁶² Both components are released into the circulation during collagen degradation and may be measured in urine by HPLC after appropriate derivatisation.⁶³ The intrinsic advantage of hydroxylysine over that of hydroxyproline is that the glycosylated forms are not metabolised and are not influenced by dietary components.^{62,63} Moreover, GGHL is present in skin and C1q, while GHL is more specific for bone. Thus the ratio of GGHL/ GHL may allow for the recognition of existent tissue specificity. Although the hydroxylysines have potential as markers of bone resorption, their major disadvantage is the current absence of a convenient immunoassay format.⁶⁴⁻⁶⁶

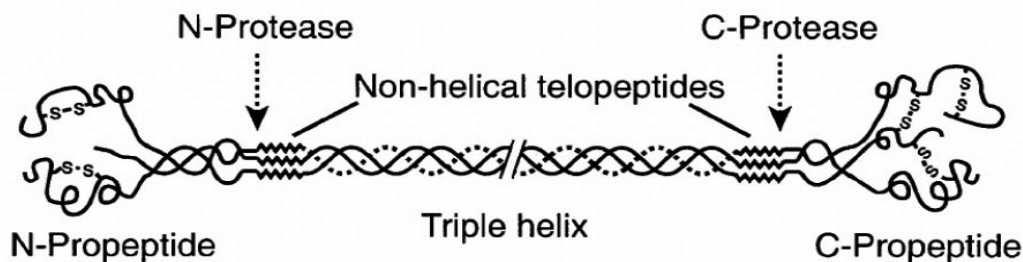


Figure 4. Schematic representation of the collagen type I molecule. The carboxy- and amino-terminal propeptides are cleaved by specific propeptidases and are partly released into the circulation. Figure courtesy of Dr Simon Robins, Aberdeen.

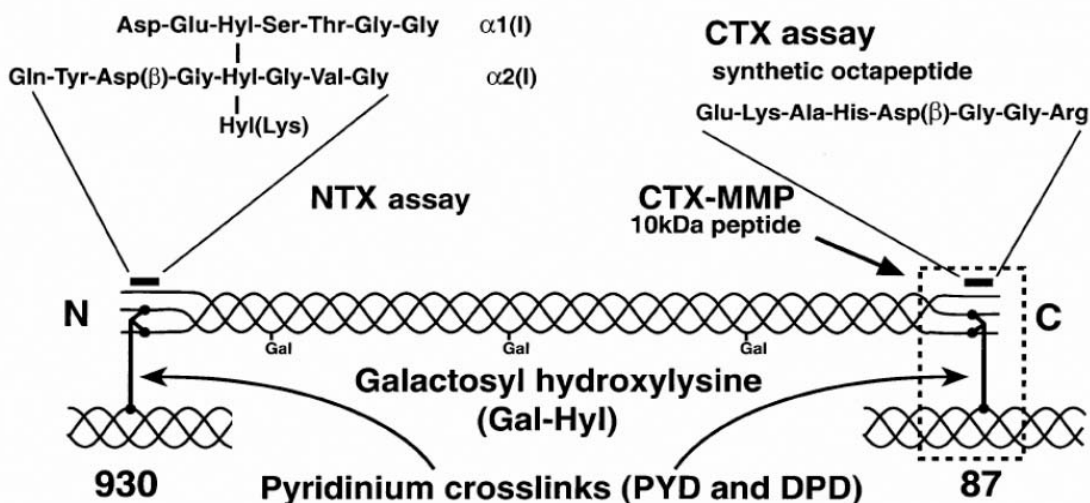


Figure 5. Molecular basis of currently used markers of type I collagen-related degradation. For more details, see text and Table 1. Figure courtesy Dr Simon Robins, Aberdeen.

3-Hydroxypyridinium Crosslinks of Collagen Pyridinoline (PYD) and Deoxypyridinoline (DPD)

PYD and DPD are formed during the extracellular maturation of fibrillar collagens. As trifunctional crosslinks, they bridge several collagen peptides and mechanically stabilise the collagen molecule (Figure 5).⁶⁷⁻⁶⁹ During bone resorption, crosslinked collagens are proteolytically broken down and the crosslink components are released into the circulation and the urine.⁷⁰⁻⁷² The measurement of hydroxypyridinium crosslinks is not influenced by the degradation of newly synthesised collagens and their levels strictly reflect the degradation of mature i.e. crosslinked collagens. In addition, the urinary excretion of pyridinium crosslinks is independent of dietary sources since neither PYD nor DPD are taken up from food.⁷³ Finally, the two hydroxypyridinium components show a high specificity for skeletal tissues. While PYD is found

in cartilage, bone, ligaments and vessels, DPD is almost exclusively found in bone and dentin. Neither derivative is present in the collagen of skin or in other sources such as C1q or elastin.^{61,74,75} Since bone has a much higher turnover than cartilage, ligaments, vessels or tendons, the amounts of PYD and DPD in serum or urine are mainly derived from the skeleton. Thus, the pyridinium crosslinks are currently viewed the best indices for assessing bone resorption.⁷⁶⁻⁷⁸

Initially, PYD and DPD were quantified in urine by reverse-phase ion-paired HPLC technique, combined with a pre-fractionation step using cellulose partition chromatography, and hydrolysis of urine samples to convert all crosslinks into the peptide free forms.^{75,79-81} Currently, automated techniques for the measurement in urine and serum are available. Analysis of urine by HPLC without initial hydrolysis showed that 40-

50% of the crosslinks were present in peptide-free form.⁸² Although the amounts of free and peptide-bound crosslinks seem to vary with the bone pathology, direct immunoassays for free and peptide-bound crosslinks are widely used. Under normal conditions, these assays have been shown to produce results similar to those provided by the traditional HPLC technique.⁸³⁻⁸⁵

Crosslinked Telopeptides of Type I Collagen

The crosslinked telopeptides of type I collagen are derived from specific regions of the collagen type I molecule, namely the aminoterminal (NTP) and the carboxyterminal (CTP) telopeptide (Figure 5).

The first collagen telopeptide assay was a RIA for the carboxyterminal type I collagen telopeptide (ICTP) in serum.⁸⁶ The respective antibodies were raised against a crosslink-containing collagen peptide (MW 8.5 kDa) isolated from human bone. The antigenic determinant requires a trivalent crosslink, including two phenylalanine-rich domains of the telopeptide region of the alpha-1 chain of type I collagen. Divalently and non-crosslinked peptides do not react with the antibody, nor do peptides isolated from skin. Despite the fact that the initial peptide contained pyridinoline, the assay also detected other crosslink forms such as deoxypyridinoline or pyrrole crosslinks. The ICTP assay appears to be sensitive for pathological bone resorption as seen in multiple myeloma, metastatic bone disease and other degradation processes involving hasty breakdown of skeletal and non-skeletal type I collagen.⁸⁷

Another group of immunoassays involve the carboxyterminal telopeptide of type I collagen, abbreviated CTX (Figure 5).⁸⁸ Employing a polyclonal antiserum against a synthetic octapeptide containing the crosslinking site, a first ELISA (termed β -CTX) recognised the C-terminal type I collagen telopeptide containing an isoaspartyl (= β -aspartyl) peptide bond in its L-enantiomeric form.⁸⁹ The β -L-aspartyl is considered to result mainly from the ageing of extracellular proteins. Only one peptide strand is necessary for immunoreactivity. Meanwhile, a monoclonal-based RIA for the non-isomerised octapeptide (EKAH- α D-GGR) in urine has been developed (" α -CTX").⁹⁰ Simultaneous measurement of both forms may be used to calculate the ratio of α -CTX / β -CTX as an index of bone turnover. This ratio has been shown to be elevated in the urine of patients with untreated Paget's disease of bone, where rapid bone formation and resorption result in an increase of α -CTX (non-isomerised octapeptide).⁹¹

Further to isomerisation, many proteins are also subjected to racemisation of certain residues. Both processes are

considered an effect of ageing, as the extent of racemisation and isomerisation increases with the time elapsed since synthesis of the protein. Additionally, antibodies directed against the D-aspartic acid residues in the CTX molecule have been described.⁹² Thus, immunoassays now exist (though not commercially available) for all four possible isomers of the CTX molecule: the native α -L form, the β -isomerised isoaspartyl peptide (β L), and the respective racemised forms α -D and β -D. The differential use of these assays may possibly provide information on the age-dependent changes of collagen in health and disease, although the clinical relevance is questionable.⁹³

More recently, a sandwich ELISA for the measurement of β -CTX in serum has been developed. When this assay was described initially, it utilised a polyclonal antiserum against collagenase-digested collagen type I together with the β -isomerised C-terminal octapeptide attached to the ELISA plate.⁹⁴ The newer assay uses two monoclonal antibodies, and it is claimed that they recognise only dipeptides containing a crosslink and two β -isomerised peptides with the same sequence as shown for the urinary assay. Although there is no direct evidence that the antigenic determinant in serum must contain a crosslink for immunoreactivity, this is indeed highly probable since β -transformation occurs slower than crosslinking, and single peptides do not react in the serum CTX-ELISA. However, the crosslink component may be any kind of crosslink, similar to the ICTP antigen in serum. Presently, standards for the original serum CTX-ELISA are made from antigens immunopurified from human urine, but automated assays (Boehringer-Mannheim) utilise synthetic antigens. Serum and urinary CTX values are highly correlated ($r = 0.86$) suggesting that the antigen is similar in both analytical media.

Yet another peptide assay measures the crosslinked N-terminal telopeptide of type I collagen. This assay (termed NTX-assay) utilises a monoclonal antibody raised against an epitope on the alpha-2 chain of type I collagen. (Figure 5).⁹⁵ However, the antibody seems to react with several crosslinking components, and the presence of a pyridinium crosslink is not essential for reactivity. As a matter of fact, digests of skin collagen exhibited similar reactivity with the NTX assay as skeletal extracts.⁹⁶ Both the monoclonal antibody and the assay format are identical for the urine and the serum based assays. As expected, the two assays show good correlation to each other, and the analyte seems to be stable at room temperature and during up to three freeze-thaw cycles.⁹⁷

Bone Sialoprotein (BSP)

BSP is a phosphorylated glycoprotein with an apparent MW of 70-80 kDa, which accounts for 5-10% of the non-collagenous

matrix of bone.^{98,99} The protein has been shown to be a major synthetic product of active osteoblasts and odontoblasts, but was also found in osteoclast-like and malignant cell lines. Consequently, BSP or its mRNA is detected mainly in mineralised tissue such as bone, dentin and at the interface of calcifying cartilage.¹⁰⁰⁻¹⁰² Intact BSP contains an Arg-Gly-Asp (RGD) integrin recognition sequence, improves the attachment of osteoblasts and osteoclasts to plastic surfaces, binds preferentially to the $\alpha 2$ chain of collagen, nucleates hydroxyapatite crystal formation in vitro and appears to enhance osteoclast mediated bone resorption.^{103,104} The protein is therefore considered to play an important role in cell-matrix-adhesion processes and in the supramolecular organisation of the extracellular matrix of mineralised tissues.

We and others have developed immunoassays for the measurement of BSP in serum.^{105,106} So far, all of these assays are based upon polyclonal antisera, and little is known about the specific nature of the respective epitopes. Antibodies do not cross-react with non-collagenous proteins such as osteonectin, fibronectin or osteocalcin.¹⁰⁶ In serum, the majority of BSP is bound to Factor H, a major regulator of the alternate complement pathway, which is found at 0.5g/L in serum. Although this phenomenon is of unknown physiological relevance, BSP/Factor H binding studies suggest that current immunoassays detect only a fraction of bioavailable BSP in serum.¹⁰⁷ Based upon clinical data and the rapid reduction of serum BSP levels following intravenous bisphosphonate treatment, it is assumed that serum BSP reflects processes mainly related to bone resorption.¹⁰⁸

Tartrate-Resistant Acid Phosphatase (TRAP, TRAcP)

This enzyme belongs to the family of the ubiquitous acid phosphatases, of which at least five different isoforms are known. These isoforms are expressed by different tissues and cells such as prostate, bone, spleen, platelets, erythrocytes, and macrophages. All acid phosphatases are inhibited by L(+)-tartrate, except band 5, which was therefore termed tartrate-resistant acid phosphatase (TRAP or TRAcP). Of the latter, 2 subforms, 5a and 5b are known, and recent research has shown that TRAP-5b is characteristic of osteoclasts.¹⁰⁹ The origin of TRAP-5a is unknown, but may be expressed by macrophages. The two isoforms 5a and 5b are different in that 5a contains sialic acid, whereas 5b does not. So far, most assays for the measurement of TRAP in blood have used colorimetry and detected both isoforms without differentiating between bands 5a and 5b. More recently, specific immunoassays for TRAP 5b have been described and clinical results indicate that this marker may be useful to assess osteoclast activity.^{110,111} The antibodies for these assays were raised against material isolated from the spleen of a patient with hairy cell leukemia or against TRAP 5b isolated from human cord plasma.^{112,113} For the conventional

TRAP assays, care should be taken after phlebotomy to stabilise the enzyme since TRAP loses more than 20% of its activity per hour at room temperature. This can be prevented by the addition of citrate buffer to the sample.¹¹⁴

Cathepsin K

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 I.^{115,116} Its clinical relevance was appreciated with the discovery that pycnodysostosis, an autosomal recessive disease characterised by osteopetrosis, was the result of mutations in the cathepsin K gene.¹¹⁷ This clinical phenotype has been confirmed in cathepsin K null mice showing dysfunctional matrix digestion.^{118,119} Immunocytochemical studies have shown that cathepsin K is located intracellularly in vesicles, granules and vacuoles throughout the cytoplasm of osteoclasts and that it is secreted into bone resorption lacunae for extracellular collagen degradation.¹²⁰ Recently, a new enzyme-linked immunoassay for measurements of cathepsin K in serum has been developed. Due to the fact that cathepsin K is expressed and secreted by osteoclasts during active bone resorption, cathepsin K, and specifically its circulating form, may be a useful and specific biochemical marker of osteoclastic activity.

Variability

Most bone turnover markers exhibit significant within-subject variability. This poses a major problem in the practical use of bone markers: Whenever a change in a bone marker level is observed in an individual patient (e.g. following an intervention), this change must be interpreted against the background of the respective marker's variability. Therefore, knowledge of the sources of variability and the strategies used to cope with "background noise" are essential for the meaningful interpretation of bone markers.

In general, non-specific variability comprises both pre-analytical (i.e. mostly subject related; CV_{PA}) and analytical (i.e. mostly assay related; CV_A) factors. Total variability is considered the sum of pre-analytical and analytical variation and defined as $CV_T^2 = CV_{PA}^2 + CV_A^2$. The ideal marker and assay is characterised by i) excellent analytical performance (i.e. high precision and accuracy) and ii) minimal and predictable pre-analytical variability. Unfortunately, no test in clinical chemistry completely meets all of these criteria. Indeed, most of the currently available assays for biochemical markers of bone turnover are characterised by substantial pre-analytical variability. The relevant pre-analytical factors affecting marker variability are summarised in Table 2 and 3.

A number of factors need to be taken into account when employing serial measurements of biochemical markers to

Table 2. Sources of pre-analytical variability.

Technical sources

Specimen and mode of sample collection
Specimen handling and storage
Thermodegradation
Photolysis
Timing of sample collection (see also diurnal variation)
Between laboratory variation

Biological (subject related) sources

Age:

 Puberty, somatic growth, menopausal transition, menopause, ageing, frailty

Gender

Ethnicity

Recent fractures (up to one year)

Pregnancy/ Lactation

Drugs:

 Anti-resorptive agent (e.g. HRT, bisphosphonates, SERMs, strontium, OPG)

 Anabolic agents (e.g. anabolic steroids, PTH, PTHrP, strontium)

 Glucocorticosteroids

 Anticonvulsants

 GnRH agonists

 Oral Contraception

Non-skeletal Disease:

 Diabetes

 Thyroid disease

 Renal impairment (GFR < 20 ml/min/1.73 m²)

 Liver disease

 Systemic inflammatory disease (IBD, RA, COPD etc.)

 Degenerative joint disease

Immobility / Loss of gravity (bed rest, space flight)

Diet

Exercise

Temporal variability:

 Diurnal (circadian)

 Menstrual

 Seasonal

Table 3. Pre-analytical and biological characteristics of bone markers.

Marker (abbreviation)	Recommended storage	Concentrations influenced by
Total alkaline phosphatase	Stable < -20°C	Liver function
Bone-Specific alkaline phosphatase	Stable < -20°C	Liver function
Osteocalcin	Unstable < -80°C	Renal function, haemolysis
Procollagen type I carboxy-terminal propeptide	Stable < -20°C	Liver function
Procollagen type I amino-terminal propeptide	Stable < -20°C	Liver function
Hydroxyproline	Stable < -20°C	Liver function, diet, inflammation (e.g. Clq)
Pyridinoline	Stable < -20°C	Liver function, active arthritis, UV radiation
Deoxypyridinoline	Stable < -20°C	Liver function, UV radiation
Carboxy-terminal crosslinked telopeptide of type I collagen (ICTP)	Stable < -20°C	Renal function, liver function
Carboxy-terminal crosslinking telopeptide of type I collagen (CTX-I)	Stable < -20°C	Renal function, liver function
Amino-terminal crosslinking telopeptide of type I collagen	Stable < -20°C	Liver function
Hydroxylysine glycosides (Hyl-glycosides)	Stable < -20°C	Liver function
Bone sialoprotein	Stable < -20°C	Renal function, Liver function, hemolysis
Tartrate resistant acid phosphatase	Unstable < -80°C	Haemolysis, blood clotting

determine changes in bone turnover. To minimise some of the limitations linked to pre-analytical and analytical variability, standardised sampling and sample handling are mandatory to obtain reliable results.

Technical Sources of Variability

In addition to parameters of assay performance (Figure 6 and 7), factors such as the choice of sample (i.e. serum versus urine), mode of sample collection (e.g. 24 hour collection versus first or second morning void), the appropriate preparation of the patient (e.g. pre-sampling diet/ fasting/ exercise before phlebotomy), the correct handling, processing and storage of specimens should always be considered. This is important as these technical sources of variability are controllable and hence modifiable. For the purpose of practical use, some technical aspects of variability are discussed in more detail in Table 2.

Thermodegradation

Some bone markers are sensitive to ambient conditions such as temperature. Thermodegradation should always be a concern with assays directed against the intact OC(1-49) molecule. Rapid enzymatic cleavage of the peptide into

smaller fragments will lead to significant signal losses if the serum sample is kept at room temperature for more than 1-2 hrs. Adding protease inhibitors will delay, but by no means prevent this process.¹²¹ Both the free and conjugated forms of PYD and DPD have been shown to be stable in urine samples kept at room temperature for several weeks. Several reports show that pyridinium crosslinks can be stored at -20°C for years and that repeated freeze-thaw cycles of urine samples have no effect on the concentrations of PYD and DPD.¹²²

Similar stability has been reported for urinary N-terminal (NTX) and C-terminal (CTX) collagen type I telopeptides, while ICTP in serum loses up to 12% of the signal when stored at room temperature for 5 days.¹²³ The stability of glycosylated hydroxylysine residues has not been fully characterised yet, but it may be necessary to add boric acid to preserve the urine samples.

The activity of serum tartrate-resistant acid phosphatase (TRAP) declines rapidly during storage at room temperature or even at -20°C but is stable when stored at -70°C or lower.¹¹⁴ Multiple freezing-thaw cycles usually have a deleterious effect on serum TRAP activity. In contrast, serum levels of BSP appear rather

stable, both at room temperature, 4°C and -20°C, and have been shown to not change significantly during repeated freeze-thaw-cycles.¹²⁴ However, when samples are being exposed to temperatures above 30°C, an increase in signal is usually seen with the RIA.

Some assays and marker components are sensitive to haemolysis of the sample, resulting in results that are either too low or too high. This is usually the case for OC and BSP, but has also been described for TRAP and some other serum markers.

Photolysis

Pyridinium crosslinks in aqueous solutions are unstable when subjected to intensive UV irradiation.^{122,123,125} The effect increases with rising pH and has been shown to be greater for free than for total pyridinoline.¹²² Urinary NTX and CTX are not affected by UV light exposure.¹²³

Timing and Mode of Sample Collection

In general, random samples can be used for measurement of most urinary parameters (but see below for diurnal variation!). For convenience, measurement of bone markers in urine is

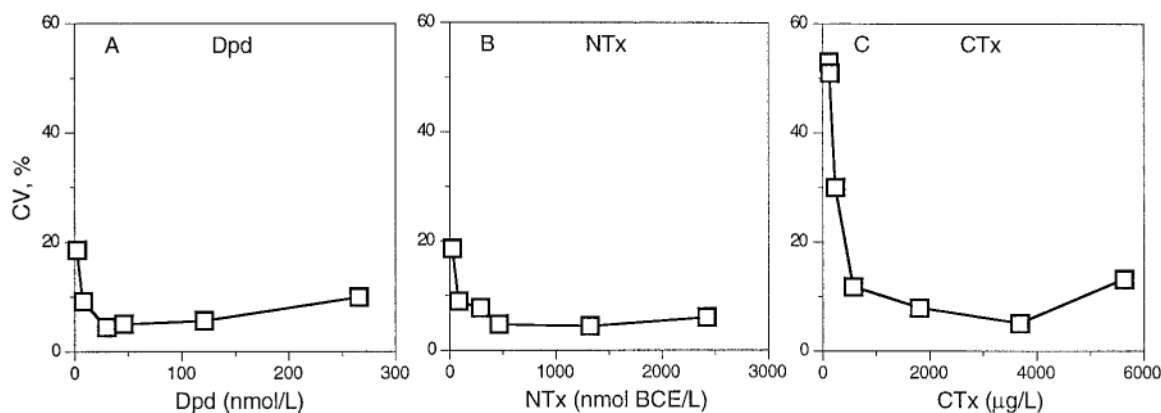


Figure 6. Intra-assay precision profiles for three immunoassays measuring type I collagen degradation products in urine. A: Deoxypyridinoline (DPD), B: Aminoterminal crosslinked telopeptide (NTX), C: Carboxyterminal octapeptide (CTX) Reproduced from, Ju HS, Leung S, Brown B, et al. Comparison of analytical performance and biological variability of three bone resorption assays. Clin Chem 1997, 43:1570-6. With permission of Clinical Chemistry.

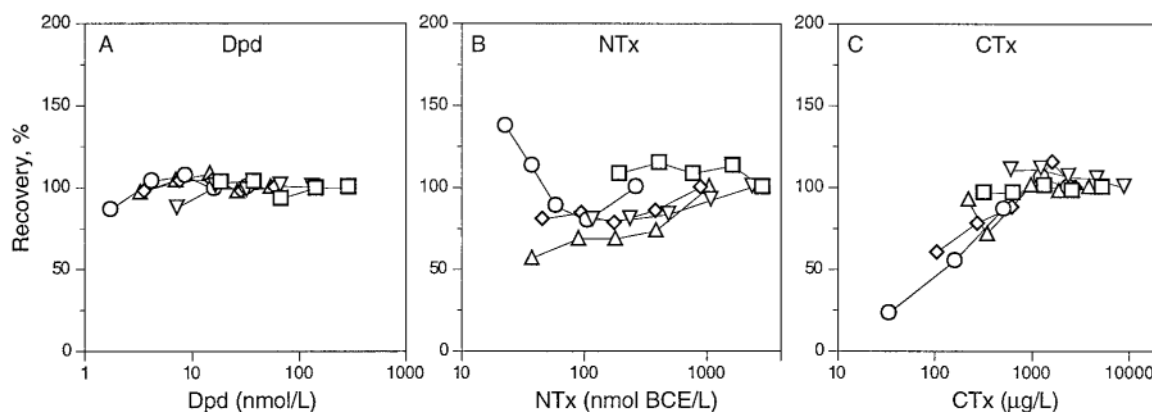


Figure 7. Dilution linearity profiles for three immunoassays measuring type I collagen degradation products in urine (n=4). A: Deoxypyridinoline (DPD), B: Aminoterminal crosslinked telopeptide (NTX), C: Carboxyterminal octapeptide (CTX). Reproduced from, Ju HS, Leung S, Brown B, et al. Comparison of analytical performance and biological variability of three bone resorption assays. Clin Chem 1997, 43:1570-6. With permission of Clinical Chemistry.

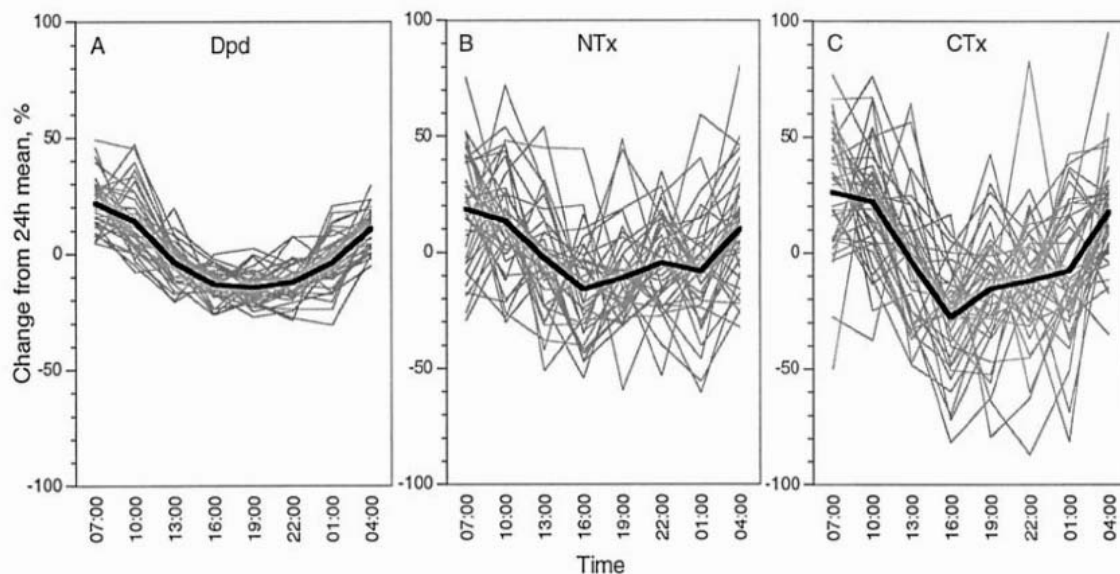


Figure 8. Diurnal variation. A-C: Comparison of three immunoassays measuring type I collagen degradation products in urine. A: Deoxypyridinoline (DPD), B: Aminoterminal crosslinked telopeptide (NTX), C: Carboxyterminal octapeptide (CTX). All values are creatinine-corrected. The thick line represents the mean, while thin lines are individual subjects. Reproduced from, Ju HS, Leung S, Brown B, et al. Comparison of analytical performance and biological variability of three bone resorption assays. *Clin Chem* 1997, 43:1570-76. With the permission of Clinical Chemistry.

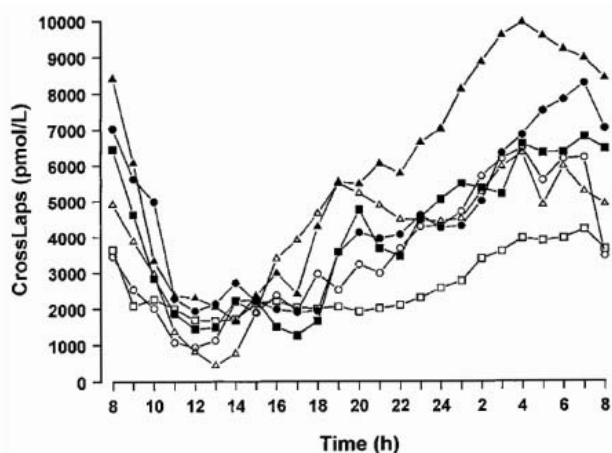


Figure 8D. Diurnal variation in serum CTX levels in six healthy male volunteers. On average, the peak value was 66% higher and the nadir was 60% lower than the calculated daily mean. Reproduced from, Wichers M, Schmidt E, Bidlingmaier F, Klingmüller D. Diurnal rhythm of Crosslaps in human serum. *Clin Chem* 1999, 45, 1858-60. With the permission of Clinical Chemistry.

usually performed either in the first or second morning void, or in 2 hour collections. In each case, values need to be corrected for urinary creatinine which introduces additional pre-analytical and analytical variability. Creatinine output has been reported to be fairly constant with time (variations

within 10%) and to correlate with lean body mass,¹²⁶ but there are also reports suggesting that the correction for creatinine in a urine spot sample could be misleading. Alternatively, the excretion rate of the marker may be determined in a 24 hour urine collection. However, these collections are subject to inevitable inaccuracies due to collection errors. With most markers, similar results are obtained from either 24 hours, 2-hours, or spot urine (FMV, SMV) collections.

It is long known that bone turnover and thus bone markers show significant diurnal variations, with highest values in the early morning hours and lowest values during the afternoon and evening.¹²⁷ Most studies report daily amplitudes of 15 – 30%, although the most pronounced diurnal changes have been communicated for CTX (Figure 8).¹²⁸⁻¹³⁴ It should be borne in mind that the slope of diurnal changes is steepest during the morning hours, which is usually the time at which urine samples are collected. This is true for both urinary and serum markers. Controlling the timing of sample collection is therefore a “bare necessity” for all types of markers (see also further below).

In addition, the effects of diet and food intake need to be considered with certain markers. For example, the ingestion of hydroxyproline-rich foods, such as meat or gelatine will markedly affect measurements of OHP in urine.¹³⁵ It is therefore necessary to instruct patients to be on a collagen-

free diet for at least 24 hours before collecting their urine for OHP measurements. In contrast, urinary and serum DPD, NTX and CTX are unaffected by collagen ingestion. Unlike most other bone markers, serum CTX values are influenced by food intake, and samples for this marker need to be taken in the fasting state.

The effect of acute exercise immediately or shortly before phlebotomy for bone turnover markers has been studied with some markers appearing to rise by as much as 30-40% of their baseline value, while others seem to be unaffected by these activities.

Variation Between Laboratories

Markers of bone turnover are now offered by a great number of commercial laboratories and in some countries are widely used by practising physicians. A recent trial amongst laboratories in Europe showed marked variability of most commercialised test kits, with inter-laboratory coefficients of variation up to 40%. Results obtained from identical blood and urine samples using the same assay and the same method differed by up to 5.6-fold between laboratories.¹³⁶ Therefore, results from different laboratories cannot be readily compared to each other, even if the same method and sample has been used. Immunoassays for bone turnover markers should be included into routine proficiency testing programs.

Biological Sources of Variability

Intra-individual (i.e. biological) sources of variability are much harder to control than technical aspects of variability. Many biological factors cannot be modified at all (e.g age, gender, ethnicity etc.), while others are hard to control in clinical practice. Nevertheless, every effort should be made to account for these factors when interpreting the results of bone marker measurements.

Effects of Age and Changes in Sex Hormone Levels on Markers of Bone Remodelling

Once somatic growth subsides, the serum and urinary concentrations of most bone markers return to a level somewhat lower than that seen during normal puberty and growth. This stabilisation usually occurs during the 3rd decade and in healthy men, levels of practically all markers remain more or less unchanged until 70 years of age. After that, a slight increase is usually seen in formation markers such as serum BAP or OC, and most resorption markers.^{84,137-139}

Menopause is associated with a substantial acceleration in bone turnover, which is mirrored by a 50-100% increase in both bone formation and bone resorption markers (Figure 9).^{137,139-148} In early postmenopausal women, this increase in bone turnover may be attenuated by calcium supplementation.¹⁴⁹

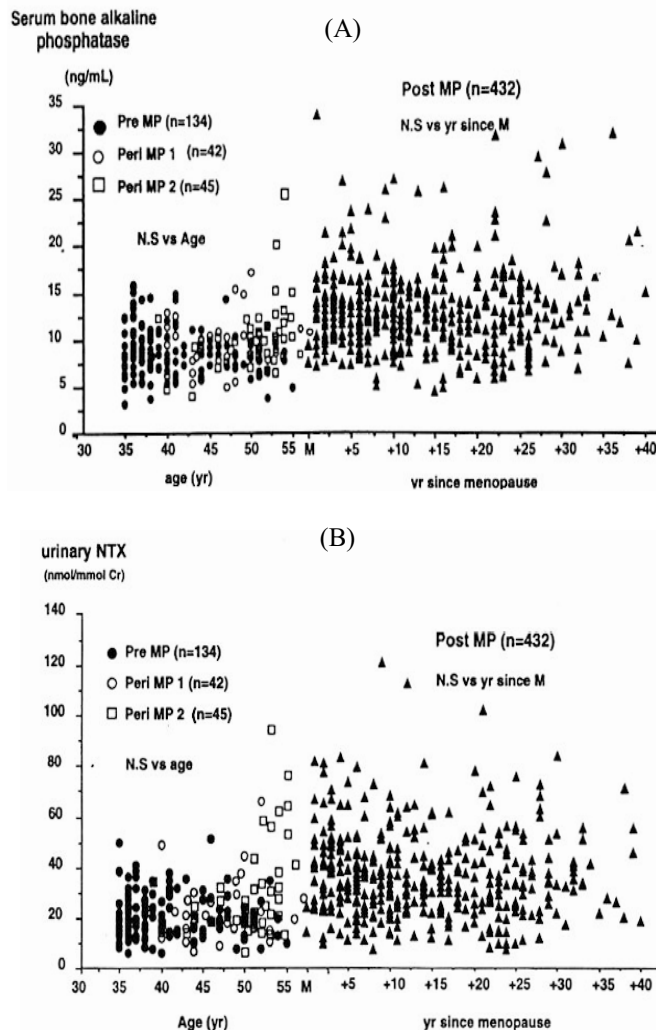


Figure 9. Age-related changes in (A) bone specific alkaline phosphatase and (B) urinary collagen type I aminoterminal crosslinked telopeptide (NTX). Pre, premenopausal women; peri MP 1, early perimenopausal women; peri MP 2, late perimenopausal women; post MP, postmenopausal women; M, menopause. Reproduced from Garnero, P, Sornay-Rendu, E, Chapuy, M-C, Delmas, PD. Increased bone turnover in late postmenopausal women is a major determinant of osteoporosis. *J. Bone Miner Res.* 1996, 11, 337-49. With the permission of the American Society for Bone and Mineral Research.

Long-term treatment of women with oestrogen has been shown to reduce resorption markers such as DPD and NTx to premenopausal levels and to correct secondary hyperparathyroidism.^{150,151} A prospective study covering the perimenopausal transition in healthy women suggests that changes in bone turnover start during late pre-menopause with a decrease in bone formation, which is later followed by a rise in bone resorption.¹⁵² It is now widely accepted that the accelerated rate of bone loss seen after the menopause is mainly due to an uncoupling in bone turnover and an increase in bone resorption. Studies employing specific bone markers

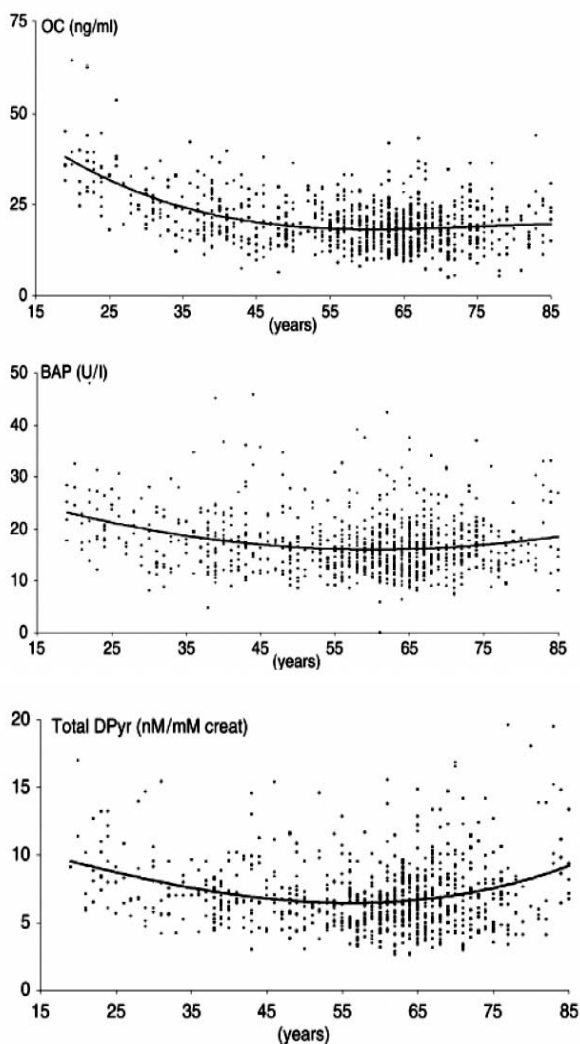


Figure 10. Age-related changes of biochemical markers of bone formation and resorption in men. (A) Serum osteocalcin, (B) Serum bone-specific alkaline phosphatase, and (C) 24-h urinary excretion of deoxypyridinoline. Reproduced from, Szulc, P, Garnero, P, Munoz, F, Marchand, F, Delmas, PD. Cross-sectional evaluation of bone metabolism in men. *J Bone Miner Res.* 2001, 16, 1642-50 with permission of the American Society for Bone and Mineral Research.

indicate that bone turnover continues to be increased (and to be associated with bone loss) even during late menopause.¹⁵³ In some postmenopausal women, but particularly in the very elderly, this increase in bone turnover is often, but not always, found to be due to vitamin D and/or calcium deficiency and secondary hyperparathyroidism.¹⁵⁴⁻¹⁵⁶

In men, the pattern of age-dependent change in bone markers is quite different from that observed in women. Markers of bone formation and resorption are high in men aged 20 to 30 years which corresponds to the late phase of formation of peak bone mass. Thereafter they decrease reaching their

lowest levels between 50 and 60 years (Figure 10).¹⁵⁷⁻¹⁶⁰ While there is general agreement on the age-related changes of bone turnover in adult men between 20 and 60 years of age, data on bone turnover rates in men over the age of 60 years are largely discordant. Based on recent cross-sectional studies, concentrations of bone formation markers remained unchanged, decreased, or increased with age in men over 60 years.^{157,158,160-163} Most studies evaluating age-related changes in bone resorption markers observed an increase in serum and urinary indices.^{157,158,160,163,164} However, this was not confirmed in other population-based studies which showed no change in resorption indices with age.^{159,165} A careful analysis of the published data provides clues that may help to explain some of these discrepancies. Differences in studies investigating age-dependent changes in bone formation and resorption may be related to diverse population characteristics and sample size, to the use of marker assays with different specificities, to age-related changes in renal and hepatic function, and lastly to the inclusion of men with osteoporosis.

As some biochemical markers of bone turnover are cleared via by the kidney (i.e. OC, collagen telopeptides), age-associated decrease in glomerular filtration rate may affect urinary and, to a larger extent, serum marker concentrations. In the presence of decreased renal function, urinary excretion of bone resorption markers expressed as 24 hour output may be falsely decreased. Conversely, levels of markers corrected by urinary creatinine can be falsely increased because of decreased creatinine filtration and decreased muscle mass.¹⁶⁶⁻¹⁶⁸

Both static and dynamic histomorphometric studies suggest that bone formation decreases with age in healthy men. In contrast, osteoclast function remains largely constant with age. Consequently, decreased bone formation seems to be the principal factor in bone loss in men.^{168,169} In the cohort of healthy men studied by Clarke et al., specific markers of bone resorption (urinary DPD) did not change with age, confirming the histomorphometric findings that osteoclast function is preserved with increasing age in these men.¹⁷⁰ These results are in contrast to some of the previously mentioned cross-sectional studies which report an age-dependent increase in serum and urinary indices of bone resorption.^{157-160,163,164,167-169}

As mentioned before, differences in population characteristics may, at least in part, explain these inconsistencies. Furthermore, men with idiopathic osteoporosis are histomorphometrically characterised by increased bone resorption with an increase in eroded surfaces up to 90% when compared with age-matched controls.¹⁷¹ Hence, the observed increase in biochemical markers of bone resorption in population-based studies might also be due to the heterogeneity of men investigated including men with osteoporosis.

Taken together it remains controversial to what extent biochemical markers of bone resorption change as a function of age in men over 60 years. Observational studies investigating age-dependent changes in bone marker levels indicate that in elderly men there seems to be an imbalance in bone turnover with increased bone resorption and stable bone formation after the age of 60. As biochemical markers have been shown to be negatively correlated with BMD, this imbalance in bone turnover may, at least in part, be responsible for the age-related bone loss in men.^{158,167} However, confounding factors such as population characteristics, specificity of bone marker, as well as estimates of renal and liver function have to be taken into account when evaluating age-dependent changes in bone turnover.

Effects of Diurnal Variation on Bone Marker Measurements

Diurnal variation appears not to be affected by age, menopause, physical activity or season. Although in postmenopausal women bone turnover is higher than in premenopausal women, the circadian variation is similar for both pre- and postmenopausal women and, thus, is not thought to be influenced by sex hormones.^{138,172,173} The aetiology of diurnal variation is unknown. Several hormones, such as parathyroid hormone, growth hormone, or cortisol show diurnal changes and may therefore be involved in the generation of diurnal changes in bone metabolism.¹⁷⁵ Independent of this, there is wide agreement that controlling the time of sampling is crucial in order to obtain clinically relevant information from bone markers. Most biochemical markers show significant diurnal variations, with highest values in the early morning hours and lowest values during the afternoon and at night. This has been well documented for most urinary markers and amplitudes usually vary between 20 and 30 % (Figure 8).¹²⁷ Serum markers usually show less pronounced changes during the day than urine based indices. However, discrepant results have been reported for serum CTX. Wichers et al. have reported daily amplitudes of serum CTX of up to 66%, while others describe smaller changes (Figure 8D).¹³⁴

Effect of Low-Frequency Biological Rhythms on Bone Marker Measurements

Intra-individually, biochemical markers of bone turnover not only vary within a single day but in most cases also between consecutive days. This phenomenon is called between-day or day-to-day variability and is apparently due to genuine variations in marker levels and not to analytical imprecision. In general, serum markers show less day-to-day variability than markers of bone turnover measured in urine.¹⁷⁵ The day-to-day variation in the urinary excretion of PYD and DPD, measured by HPLC and corrected for creatinine, ranges between 16-26%.¹⁷⁶ Similar results have been reported for free pyridinoline by EIA (7-25%), for the N-

terminal collagen type I telopeptide (NTX, 13-35%), for the C-terminal collagen type I telopeptide (CTX, 12-35%) and for tartrate-resistant acid phosphatase (TRAP, 10-12%).^{175,177} Day-to-day variability adds considerably to the total variation of biochemical markers of bone turnover and unlike diurnal variation, cannot be controlled.

Bone turnover varies with the menstrual cycle with an overall amplitude of approximately 10-20%.^{178,179} There is evidence to support the suggestion that bone formation is higher during the luteal than the follicular phase, whereas bone resorption is higher during the mid-follicular, late-follicular and early luteal phase.^{179,181} Cyclical changes in bone turnover have also been reported in postmenopausal women treated with sequential oestrogen/gestagen regimens, showing decreases during oestrogen treatment and increases during gestagen treatment.¹⁸² In premenopausal women with metabolic bone disease, menstrual variability should be taken into account, and the timing for sampling is probably best during the first 3-7 days of the menstrual cycle.

Bone turnover and its regulation seem to vary with seasonal changes. Some studies have shown that serum 25-OH vitamin D and urinary calcium are higher in summer than in winter, and that parathyroid hormone levels show inverse changes.^{182,183} More recently, seasonal changes were also described for markers of bone metabolism, with a 20-30 % lower turnover rate in summer than in winter (Figure 11).¹⁸⁴ The increase in bone turnover in winter may be due, at least in part, to subclinical vitamin D deficiency.

Effects of Somatic Growth

During early childhood and then again during the pubertal growth spurt, biochemical markers of bone turnover are significantly higher than during adulthood.¹⁸⁸ In girls, peak bone marker levels are observed approximately two years earlier than in boys, and oestradiol seems to be the major determinant of the increase in bone turnover. In men between 20 and 30 years of age, bone turnover markers are usually higher than in women of the same age bracket. After the age of 50, most bone turnover markers tend to increase with further ageing, but less in men than in women. In the latter, the age-related increase in bone turnover is more pronounced due to the menopause, when both bone resorption and formation increase by about 50-100 %.^{186,187}

Other Biological Sources of Variability

A number of non-skeletal diseases have been shown to strongly affect bone turnover markers. These conditions mostly relate to impairments in the clearance and / or metabolism of the components measured. Thus, even moderate impairment of renal function (GFR 50 ml/min) has been shown to have

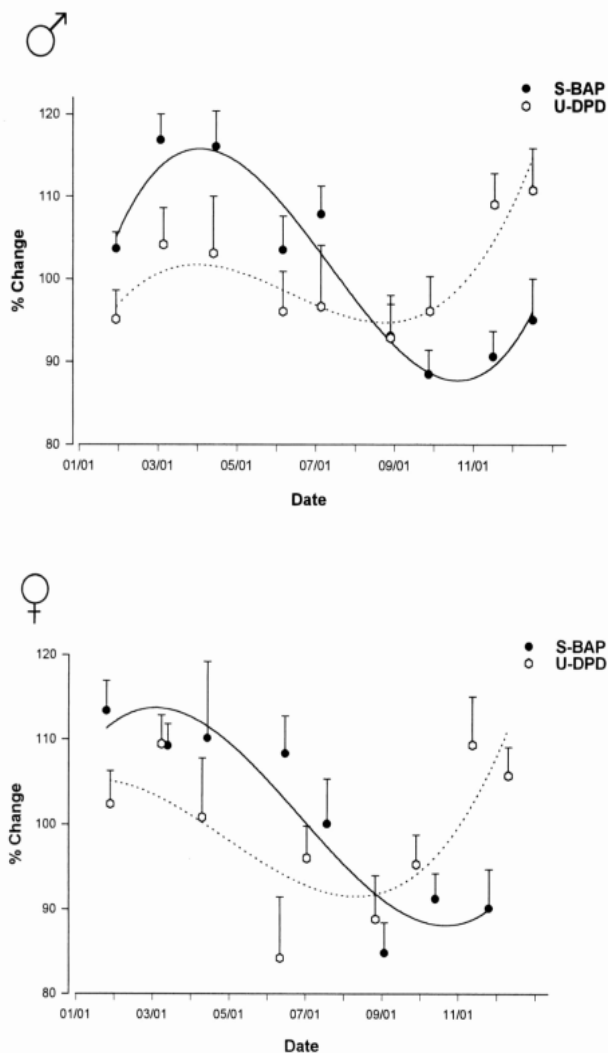


Figure 11. Seasonal variability of serum bone specific alkaline phosphatase (S-BAP) and urinary deoxypyridinoline (U-DPD). Values are presented as the percent change from the annual mean (\pm SEM). The number and width of representative intervals of each bone marker were computed as $\Delta = (D_{\max} - D_{\min}) / (1 + 3.322 \times I_g n)$, in which Δ is the interval width, D_{\max} is the end of the analysis period (December 31), D_{\min} is the beginning of the analysis period (January 1), n is the number of data points, and $1 + 3.322 \times I_g n$ gives the number of intervals. After computing mean values and weighing the number of data points in each interval, graphs were constructed using the least square curve fitting by means of a polynomial regression. Data points (mean values \pm SEM) are placed on the median of the respective interval (i.e. for S-BAP in males, January 25 represents the median of the first interval ranging from January 1 to February 15). Differences in the number and width of representative intervals between parameters result from missing laboratory values or elimination of extreme values in some instances. Reproduced from, Woitge, H, Scheidt-Nave, C, Kissling, C, et al. Seasonal variation of biochemical indices of bone turnover: results of a population based study. *J Clin Endocrinol Metab.* 1998;83:68-75. With permission from The Endocrine Society.

significant effects on the serum levels of OC, of BSP, and of the collagen type I telopeptides (NTX, CTX).^{108,188,189}

In summary, numerous factors influence bone turnover, but there are even more sources of variability that need to be taken into account when measuring biochemical markers of bone turnover. To minimise some of the limitations linked to pre-analytical and analytical variability, standardised sampling and sample handling are mandatory to obtain reliable results. Controllable factors such as the mode of sample collection, sample handling and storage, diurnal and menstrual rhythms, pre-sampling exercise and pre-sampling diet should be taken care of wherever possible. Laboratories are encouraged to establish their own reference ranges and to use gender and age specific reference intervals. In order to further reduce variability, standardisation of bone marker assays and routine proficiency testing programs are strongly recommended.

How to Deal with Variability

If bone markers vary so much, how can we ever use them in our patients? Admittedly, most data on the use of bone markers have been generated in large populations or controlled clinical trials, which may not be representative of a routine clinical setting. However, bone markers can well be used in individual patients if methods are used that account for potential sources of variability and thus integrate the inherent limitations of bone markers measurements.

The Concept of Least Significant Change (LSC)

Numerous biological factors affect bone turnover and therefore bone marker levels (Table 2). As a rule, markers showing large changes in response to disease processes or interventions also show substantial degrees of biological variability. In the clinical setting, variability of bone markers should be of particular concern when it comes to serial measurements, for example during therapeutic monitoring. Often, a moderate reduction in a bone resorption marker is believed to be the effect of anti-resorptive treatment, when it really should be attributed to non-specific variability. However, a true ("significant") response in either BMD or bone turnover can only be assumed, when within a single individual the change in signal is greater than the imprecision of the measurement. This change is called the "LSC". LSC can be defined for various levels of confidence (e.g. 80% or 95%) and in large depends on the short- and long-term within subject variability (CV) of a given marker. The CV of bone formation markers is lower than that of most bone resorption markers, and so is their LSC. Thus, for formation markers, a change $>25\%$ should under regular circumstances be considered significant, while for most bone resorption markers (serum and urine) LSC is around 60-80%.

Another phenomenon to be considered when interpreting any serial measurement is the regression to the mean. This effect is independent from biological variability and relates to the changes in extreme baseline values.

The pronounced variability and heterogeneity of bone markers makes it difficult to determine precise thresholds or cut-offs for practical use in individual patients. In clinical medicine, two interrelated approaches are often used to assess the clinical significance of a change. These include a comparison of the actual difference to a predefined cut-off, and a comparison of the measured value to a predefined range.

The first method defines certain cut-off levels that a change in a marker must exceed to be considered “clinically significant”. Some models apply LSC or similar statistical approaches to define *a-priori* cut-offs.¹⁹⁰ Other models have used the placebo and treatment groups of large randomised controlled trials (RCTs) and calculated the fracture incidence according to the change in a marker above or below a certain cut-off. For example, using data from the fracture intervention trial, Bauer et al. demonstrated that alendronate-treated women with a reduction in serum BALP of 30% or more at 1 year had fewer hip and non-spine (but not vertebral) fractures than alendronate-treated women with a 1 year change in serum BALP of less than 30%. However, when compared to the *placebo* group, there was no difference in the incidence of vertebral fractures between the alendronate-treated groups (>30% vs. <30% change at 1 year). In this instance, therefore, setting a 30% cut-off helped to define – *a posteriori* – a valid

response to alendronate treatment for non-spine and hip fractures, but appears to be useless for vertebral fractures.¹⁹⁴

Another widely used method to assess therapy-induced changes in laboratory parameters is to compare the actual marker level to a predefined range (similar to the T and Z score approach in bone mineral density [BMD]). In most instances, this range will be the ‘reference’ or ‘normal’ range, which may or may not be standardised between methods and/or laboratories. In the bone field, most people would agree that patients with accelerated bone turnover are likely to benefit from anti-resorptive treatment if their bone markers return to the respective ‘normal’, i.e. premenopausal range. Such a ‘normalisation’ would be considered a valid response, while changes that do not lead to a return of the marker into the reference range would be labelled as an invalid response. Furthermore, a reduction of the marker below the reference range could be indicative of over-treatment.

A recent paper by Sambrook et al. illustrates the usefulness of this approach. In a double masked and double placebo controlled study, the authors compared the effects of alendronate 70 mg once weekly with raloxifene 60mg daily on markers of bone turnover in 487 postmenopausal women with low BMD.¹⁹³ Both anti-resorptive agents reduced serum OC and urinary NTX-I levels after 1 year of treatment, but the effect was more pronounced for alendronate. Large RCTs have shown that both alendronate and raloxifene reduce fracture risk in women with low BMD. It is therefore interesting to note that in the head-to-head trial by Sambrook et al., both

List of Abbreviations

TAP	Serum total alkaline phosphatase
BAP or Bone ALP	Serum bone alkaline phosphatase
BMD	Bone mineral density
BSP	Bone sialoprotein
β-CTX-I	Epitope of C-terminal cross-linked telopeptide of collagen type I
DPD	Deoxypyridinoline
ICTP	Epitope of C-terminal cross-linked telopeptide of collagen type I
LSC	Least significant change
NTX-I	N-terminal cross-linked telopeptide of collagen type I
PICP	C-terminal propeptide of procollagen type I
PINP	N-terminal propeptide of procollagen type I
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
PYD	Pyridinoline
TRAP or TRAcP	Tartrate-resistant acid phosphatase

alendronate and raloxifene returned bone markers into their respective reference ranges.¹⁹²

The problem with this method is that reference ranges for most markers have not been well defined. Also, a reduction into the 'normal' range can only be achieved if the pre-treatment values are abnormally high, which is the case in less than 50% of patients with osteoporosis. Clearly, the approach is also invalid for anabolic treatments, which increase both bone formation and resorption. Finally, standardisation of most assays is presently insufficient to provide a basis for a wider application of such a reference-based approach.¹³⁶

Competing interests: None declared

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