



## Review

# Bone markers

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### Abstract

**Objectives:** Bone has a dynamic metabolism that includes modeling and remodeling activities. There is continuous communication between 3 types of bone cells; osteoblasts, osteoclasts, and osteocytes. Local stress factors, cytokines, and hormones play an important role in these relationships. The most important structural component of bone is type 1 collagen. During the formation and degradation of collagen, some compounds are secreted into the bloodstream, and in cases of diseases involving the bone, the quantity of these compounds increases both in blood concentration and urinary excretion. Some bone markers are secreted into the circulation during bone formation, and some are released into the circulation through bone resolution. Bone markers reflect changes in bone metabolism due to primary or secondary causes, rather than a specific bone disease. Some factors affecting the results should be considered during the evaluation of changes. These factors include preanalytical effects, such as age, gender, diurnal rhythm, and analytical problems. This review is a summary of the current applications of bone turnover markers and the effects of preanalytical conditions.

**Keywords:** Bone biochemistry, bone cells, bone marker

**B**one tissue is one of the hardest tissues in the body. It has 3 important functions: mechanical, protective, and metabolic. The mechanical functions of the skeletal muscles are to provide body movement based on attachment to the bones. Contraction allows body movement. The protective function of the bones is the armor provided to internal organs, such as those in the cranium and thorax. Finally, there is the process of hematopoiesis in the bone marrow. The metabolic function of bone provides for the storage of ions, such as calcium, phosphorus, sodium, and magnesium, and the maintenance of hemostasis of these minerals [1-3]. This paper is a discussion of new and novel bone markers and preanalytical factors that affect analytical methods.

### Bone tissue structure

Bone is a mineralized connective tissue composed of an organic and inorganic structure. The inorganic structure, or mineral structure, of bone is primarily hydroxyapatite  $\text{Ca}_{10}$

$(\text{PO}_4)_6(\text{OH})_2$  crystals, as well as magnesium, carbonate, and fluoride. The majority of calcium in the body is in the bones (about 99%). Hydroxyapatite crystals provide resistance to bone [4, 5]. The collagen fibrils are the element that provides strength, while hydroxyapatite crystals contribute hardness. The organic matrix forms about half of the dry weight of the bone. Collagen is the major protein of the organic matrix; non-collagen molecular (glycosaminoglycans and glycoproteins, etc.) constitute about 10% of the organic matrix. Although 80% to 90% of collagen is type 1 collagen, other collagen types (type 3, 5, 11, 13 collagen) also make up the matrix structure [2, 6, 7]. Non-collagen proteins, such as proteoglycans (chondroitin sulfate and proteoglycan), glycoproteins (alkaline phosphatase and osteonectin), glycoproteins containing arginine-glycine-asparagine, (osteopontin and bone sialoprotein), osteopontin, and carboxylated (Gla) proteins (osteocalcin and matrix Gla protein), are also present [8, 9].

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**Submitted Date:** April 24, 2019 **Accepted Date:** April 27, 2019 **Available Online Date:** June 12, 2019

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### Bone cells and bone turnover

Bone is a metabolically active tissue that is constantly renewed by resorption, formation, and remodeling. The annual regeneration rate of bone in a healthy adult is approximately 10%. The cells responsible for bone resorption are osteoclasts, and the cells involved in bone structure are osteoblasts [10, 11]. The functions of these cells are summarized in Table 1. Osteocytes develop from osteoblasts and also contribute to the construction of bone matrix [12, 13]. Osteocytes express receptors for many hormones and cytokines. Due to the secretion of sclerostin and fibroblast growth factor 23 (FGF23), it acts as an endocrine cell. These factors are important for osteocyte-osteoblast interactions. In the first phase of reconstruction, osteoclasts perform bone resorption. During bone resorption, acid and hydrolytic enzymes are secreted from osteoclasts. Osteoclastic degradation of the bone matrix leads to a release of bone minerals and fragments of collagen. Although some collagen is incompletely hydrolyzed, resulting in the formation of pyridinoline cross-links bound to fragments of the alpha-1 and alpha-2 chains of collagen, the majority of the collagen is completely hydrolyzed to its small units, such as pyridinoline (PYD) and deoxypyridinoline (DPY). In the second stage, osteoblasts form the bone matrix, followed by a mineralization phase [14-17].

Type 1 collagen is necessary for mineralization [18]. Collagen is a protein that forms a triple helix structure. Procollagen is the formation of the first helical structure during collagen synthesis. The amino and carboxyl peptides present at both ends of the procollagen molecule are removed from the structure

by proteases during collagen synthesis, resulting in the formation of tropocollagen. Tropocollagen contains portions at each end that do not demonstrate a helix structure: The nonhelical portions at the two terminals of tropocollagen are the N-telopeptide and C-telopeptide regions. Cross-links are formed between lysine and/or hydroxylysine side chains of tropocollagen [2, 19]. This cross-linking is affected by a copper-dependent enzyme, lysyl oxidase. Cross-linking takes place between lysine and hydroxylysine in tropocollagen molecules. PYD and DPY are cross-links. The PYD cross-linking region in the N-telopeptide region is the amino-terminal telopeptide (NTX). The carboxy-terminal telopeptide (CTX) is an isomerized fragment in the C-terminal region [2, 6, 7, 18, 19].

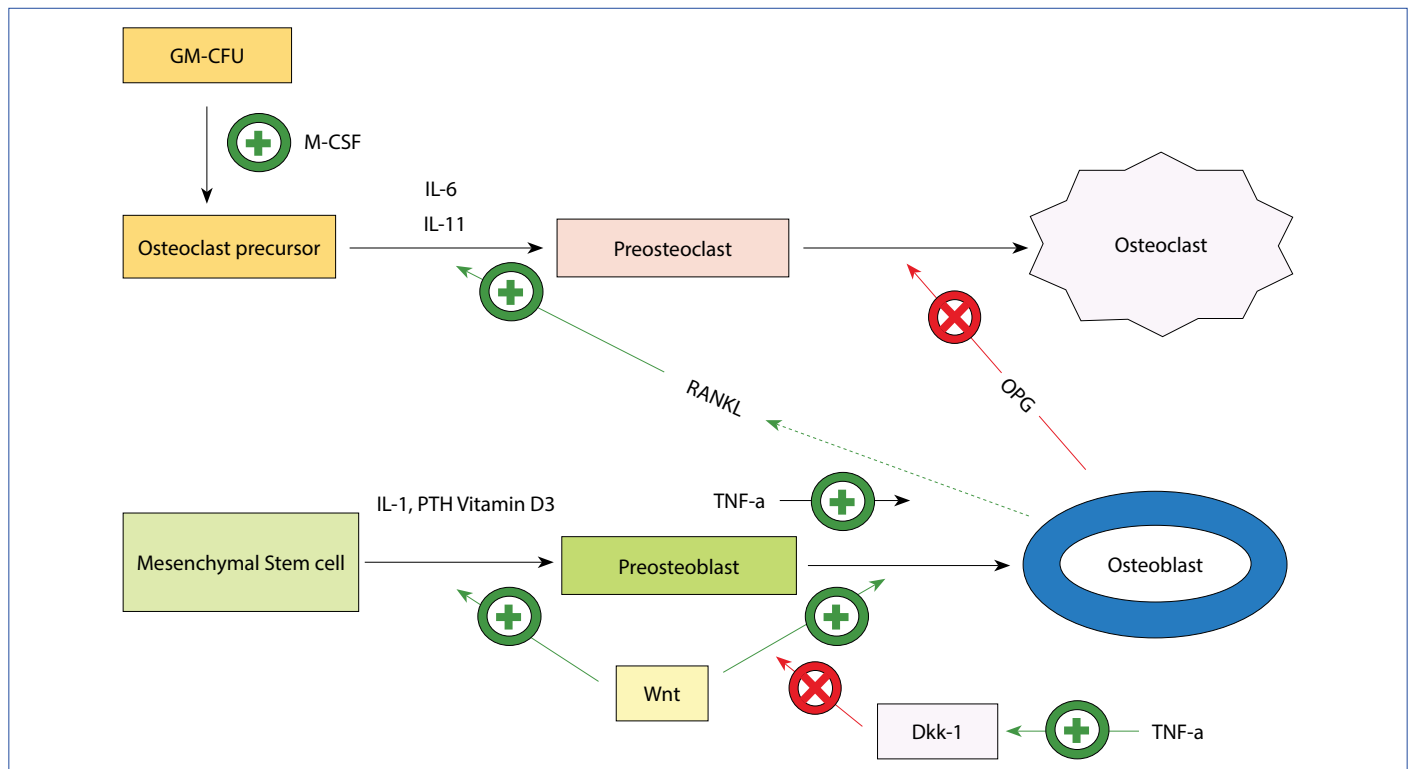
Bone resorption and formation are determined by molecules that affect osteoblast or osteoclast activity [10-12]. Osteoblasts regulate bone resorption through the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), as well as bone formation [20]. RANKL is a ligand for the receptor activator of the nuclear factor kappa-B (RANK) receptor and is responsible for stimulating resorption via the formation and activation of osteoclasts. Osteoblasts also form osteoprotegerin (OPG), a soluble receptor. OPG impedes bone resorption by inhibiting the differentiation and proliferation of osteoclasts. This occurs through blocking the interaction of RANKL with its receptor, RANK, which is localized on the osteoclasts. In summary, OPG and RANKL are synthesized by osteoblasts, and are involved in osteoblast-osteoclast interaction [20-22]. The net effect of OPG and RANK is the regulation of osteoclast activation and thus, bone resorption (Fig. 1).

**Table 1. Bone cells and main functions**

Cell	Characteristics	Function and roles in bone remodeling
Osteoblasts	Osteoblasts are bone-forming cells derived from pluripotent precursors. They synthesize many proteins, growth factors, and cytokines in the bone.	Osteoblasts are responsible for the construction of new bone matrix and mineralization. They control mineralization by regulating the transition of calcium and phosphate ions from the surface membranes. The cell surface contains parathormone, D-vitamin, and estrogen receptors. Alkaline phosphatase enzyme is present in the plasma membrane.
Osteocytes	Osteocytes are the most abundant cells in the bone. They are a type of osteoblast that reduces metabolic activity and resorbs bone. They form when an osteoblast becomes embedded in the matrix it has secreted.	Osteocytes keep bone tissue alive. Nutrients and hormones pass from cell to cell with their cytoplasmic extensions. They are involved in sensing the mechanical load applied to bone and biochemical signalling leading to resorption or formation. They are responsible for providing phosphate homeostasis by regenerating the mineralized matrix and regulating the excretion of enough calcium to the circulation and phosphate from the kidneys.
Osteoclasts	Osteoclasts are multiple nucleus degradation cells derived from pluripotent hematopoietic stem cells. They have an apical membrane that acts as a key to bone resorption.	Calcitonin receptors are present in the osteoclast membrane. There are no parathyroid hormone or D vitamin receptors. Osteoclasts include tartrate-resistant acid phosphatase and carbonic anhydrase. Osteoclasts reduce pH via the membrane-based ATPase enzyme. Hydroxyapatite becomes soluble and demineralization occurs in the bone.

**Table 2. The main hormones that affect bone metabolism**

Hormone	Effects
Parathyroid hormone	Parathyroid hormone causes resorption of bone tissue. <ul style="list-style-type: none"> <li>• Provides the release of calcium and phosphorus</li> <li>• Restricts collagen synthesis in osteoblasts</li> <li>• Stimulates bone resorption in mature osteocytes</li> <li>• Causes solubility in osteoclasts</li> <li>• Allows early transformation of cells into osteoclasts and osteoblasts</li> <li>• Reduces calcium binding capacity of bone</li> </ul>
Calcitonin	Calcitonin acts in the opposite way of parathyroid hormone. Calcitonin inhibits bone resorption in pharmacological doses by inhibiting osteoclasts.
Sex hormones	Estrogen decreases osteoid matrix production and increases bone resorption due to estrogen deficiency during menopause. Testosterone shows estrogen-like effects.
Growth hormones	Growth hormones are important for skeletal growth. They provide positive calcium-phosphate balance.
Thyroid hormones	Thyroid hormones stimulate both bone resorption and formation. Thus, hyperthyroidism accelerates bone turnover.
Steroids	Steroids directly stimulate bone destruction. It may be due to accelerated apoptosis of osteoblasts and osteocytes.
Prolactin	Prolactin accelerates bone loss by suppressing estrogen and testosterone production.



**Figure 1.** Differentiation of bone cells: Role of RANKL and OPG. ILs, PTH, and vitamin D3 are related to bone cell formation. Bone cells interact with each other. RANKL and OPG from the osteoblast regulate osteoclast formation. DKK-1 is a regulator of osteoblast activity via the Wnt pathway. It is a negative regulator of Wnt signaling. Wnt signaling is associated with control of cell proliferation as well as osteoblasts.

Dkk-1: Dickkopf-related protein 1; GM-CFU: Granulocyte-macrophage colony-forming unit; IL: Interleukin; M-CSF: Macrophage colony-stimulating factor; OPG: Osteoprotegerin; PTH: Parathyroid hormone; RANKL: Receptor activator of nuclear factor kappa-B ligand; TNF- $\alpha$ : Tumor necrosis factor alpha.

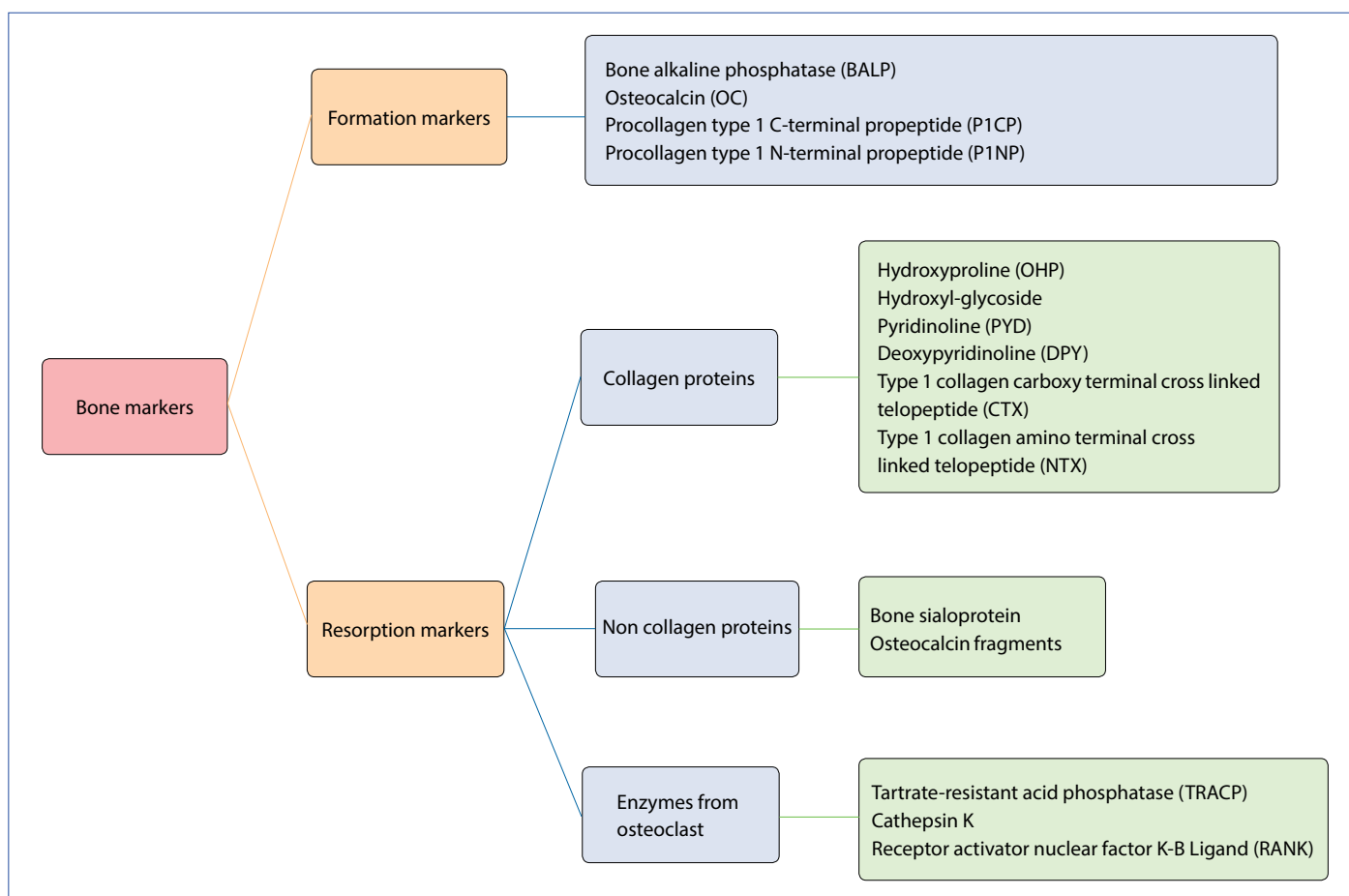
Several hormones are involved in the regulation of bone metabolism [23-26]. Osteoblasts are stimulated by parathyroid hormone and vitamin D, but are inhibited by corticosteroids. While

parathyroid hormone and vitamin D also stimulate osteoclasts, calcitonin and estrogen inhibit the activities of osteoclasts. Table 2 illustrates the hormones that affect bone metabolism.

**Table 3. Serum and urinary bone turnover markers and assay methods**

Marker	Activity	Sample	Method (s)
Bone-specific alkaline phosphatase	Formation	S, P	E, IRMA, EIA
Osteocalcin	Formation	S	RIA, ELISA, IRMA, ECLIA
Procollagen type 1 C-terminal propeptide	Formation	S	RIA, ELISA
Procollagen type 1 N-terminal propeptide	Formation	S, P	RIA, ELISA
Hydroxyproline	Resorption	U	HPLC, C
Hydroxylysine-glycosides	Resorption	U (S)	HPLC, ELISA
Pyridinoline	Resorption	U, S	HPLC, ELISA
Deoxypyridinoline	Resorption	U, S	HPLC, ELISA
Type 1 collagen carboxy-terminal cross-linked telopeptide	Resorption	U, S	ELISA, RIA, ECLIA
Type 1 collagen amino-terminal cross-linked telopeptide	Resorption	U, S	ELISA, RIA, ICMA
Bone sialoprotein	Resorption	S	RIA, ELISA
Osteocalcin fragment	Resorption	U	ELISA
Tartrate-resistant acid phosphatase	Resorption	P, S	RIA, ELISA, C
Cathepsins	Resorption	P, S	ELISA

C: Calorimetry; E: Electrophoresis; ECLIA: Electrochemiluminescence immunoassay; ELISA: Enzyme-linked immunoassay; HPLC: High-performance liquid chromatography; ICMA: Immunochemiluminometric assay; IRMA: Immunoradiometric assay; P: Plasma; RIA: Radioimmune assay; S: Serum; U: Urine.

**Figure 2.** Classification of bone turnover markers.

The final phase of the process of bone turnover is the resting phase. In healthy individuals, bone formation matches bone destruction and there is no loss in total bone mass. In cases

of metabolic bone disease, the balance between bone resorption and formation is impaired; bone resorption and/or bone formation increases or decreases. The remodeling processes

of bone has demonstrated adaptation to local and environmental stimuli (physical or hormonal) [27, 28].

### Bone turnover markers

The metabolic status of bone can be evaluated using a group of molecules called bone turnover markers (BTMs). BTMs consist of estrogens and structural proteins released from the collagen matrix [28, 29]. Although BTMs are divided into 2 groups, formation and resorption markers, some markers allow for evaluation of both formation and resorption (Fig. 2). Bone resorption markers indicate type 1 collagen degradation and osteoclast activation. Type 1 procollagen products and molecules expressed from activated osteoblasts are defined as markers of bone formation. In bone disorders, bone metabolism is dramatically altered, and either class of marker will identify changes in bone turnover. If the clinical situation has influenced the development stage of osteoblasts, BTM mismatches will occur [30-32].

BTMs are assayed in serum and/or urine using several methods, such as electrophoresis, radioimmunoassay, high performance liquid chromatography, enzyme immunoassay, and colorimetric assay (Table 3). Commonly used bone markers are summarized below.

### Bone formation markers

**Bone-specific alkaline phosphatase (BALP):** Alkaline phosphatase (ALP), a membrane-bound enzyme, has 4 isoforms, which are located in the bone, placenta, liver, and intestine. The bone and liver isoforms are the most common (>95%) in circulation [33]. BALP is produced by osteoblasts and elevated BALP levels are positively correlated with bone formation rate. BALP also plays a key role in degrading the natural inhibitor of mineralization pyrophosphate [30-32, 34].

**Osteocalcin (OC):** OC, a calcium-binding peptide, consists of 49 amino acids. OC is expressed and secreted by osteoblasts [8, 10]. Most synthesized OC enters the bone matrix, but a small amount is released into the blood. Vitamin K and 1.25-dihydroxyvitamin D are necessary for OC synthesis [35, 36]. During synthesis, vitamin K dependent carboxylation occurs in specific glutamate residues of molecules. This posttranslational modification gives the protein the ability to bind to calcium. OC synthesis is induced by vitamin D. There are also carboxylated forms of OC in the blood, as well as non-carboxylated forms. OC, which has a very short half-life, breaks down rapidly and forms OC fragments. In some cases, it is thought that these fragments may be a source of information about bone metabolism. Although it is accepted as a bone formation marker, during bone resorption OC can be liberated. Therefore, the net effect is still uncertain in various clinical situations. In addition, due to difficulties of analysis, stability problems in the test sample, and a high degree of biological variation, OC provides only limited information about bone metabolism. [37-39]. In summary, serum OC levels provide important information about osteoblastic activity, rather than showing the severity of bone disorders.

**Procollagen propeptides:** Procollagen is the precursor of type 1 collagen. The procollagen molecule has amino and carboxyl terminal extensions. During synthesis, these extensions are enzymatically removed from the procollagen molecule and released into the circulatory system. These peptides are the procollagen type 1 C-terminal propeptide (P1CP) and the procollagen type 1 N-terminal propeptide (P1NP). Although these procollagen peptides are specific to type 1 collagen rather than bone, the concentration is reduced by estrogen and anti-resorptive therapy and increased by parathyroid hormone therapy [40-45]. It has been suggested that PINP in the blood may be one of the reference markers of bone turnover for fracture risk prediction and monitoring of osteoporosis treatment [46, 47]. However, it has also been noted that pre-analytical variances affected P1NP measurements. Although P1NP has minimal circadian variability, sample instability has been observed [48, 49].

### Bone resorption markers

**Collagen cross-links:** PYD and DPY are cross-linking collagen polypeptide chains that provide stabilization of collagen [6, 7]. The most important function of the cross-linking pattern is that it provides the mechanical properties of type 1 collagen. Collagen cross-linking also affects the differentiation of osteoblasts [49, 50]. Cross-linkages formed during extracellular collagen maturation are released into the blood as a result of mature collagen degradation [6, 7]. PYD and DPY are found in urine in both free and peptide-bound forms.

PYD is found in bone as well as in cartilage, whereas DPY is specific to the bone and dentin. Therefore, DPY is a more specific and sensitive bone marker than PYD. Collagen cross-linkages can be useful in clinical conditions, especially when bone resorption is critical, such as in osteoporosis and osteoarthritis [51, 52].

**Hydroxyproline (OHP):** OHP is derived from the post-translational hydroxylation of proline [2, 6, 7]. The presence of OHP increases the elasticity of collagen. Proline reduces the elasticity of collagen. There are 2 sources of circulating OHP: dietary intake and bone resorption. OHP enters the circulation during bone destruction and collagen degradation. Gene mutations leading to a change in the proline and/or OHP content of collagen lead to diseases in which collagen elasticity is affected [53]. Therefore, OHP is a marker of collagen degradation rather than a BTM. It has been reported that prolyl-hydroxyproline, as a collagen-derived dipeptide, was associated with osteoblast differentiation [54, 55].

**Telopeptides of type 1 collagens (CTX, NTX):** NTX and CTX are released during collagen degradation [6, 7]. The International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine Working Group have suggested that the blood level of CTX and NTX may be a bone resorption marker [46-48]. Serum NTX and CTX levels have been reported to be elevated in cases of bone metastasis and in postmenopausal women [56, 57].

**Tartrate-resistant acid phosphatase (TRACP):** TRACP is a lysosomal enzyme [58, 59]. It has 2 isoforms in the circulation: TRACP5a found in activated macrophages and TRACP5b derived from osteoclasts. During bone resorption, TRACP5b is secreted by osteoclasts and generates reactive oxygen species. Therefore, it is accepted as a marker of both the number of osteoclasts and a measure of activity. The fact that renal dysfunction or diet does not affect TRACP5b levels is important for clinical use of this marker [60, 61].

**Hydroxylysine-glycosides:** Hydrogen lysine, which is present in the structure of collagen, is formed by post-translational modification during collagen synthesis. The lysyl hydroxylase enzyme provides for the formation of hydroxylysine. Another modification of collagen is the transfer of galactose to these hydroxylysine derivatives. This glycosylation reaction results in galactosyl hydroxylysine (GHL). During collagen breakdown, hydroxylysine and GHL are circulated and excreted in the urine. It is an important advantage that hydroxylysine is not affected by diet and that the only source of GHL is bone. Therefore, collagen degradation is more specific than OHP [62, 63].

#### Other bone matrix proteins

**Osteonectin:** Osteonectin is one of the noncollagenous calcium-binding glycoproteins in bone. It is also a secreted protein acidic and rich in cysteine (SPARC). Osteonectin mediates the maintenance of bone mass and normal remodeling. It has been reported that decreased osteonectin levels were related to a low number of osteoblasts and bone formation rate. SPARC mutations have been identified in patients with idiopathic osteoporosis [64, 65]. However, osteonectin is also found in non-bone connective tissue and platelets. Therefore, it is not a bone-specific biomarker.

**Bone sialoprotein (BSP):** BSP is a glycoprotein of the bone matrix. It is one of the non-collagen phosphorylated proteins in bone. It is only found in mineral tissue and is produced by bone cells. BSP is important for cell matrix adhesion and also has an activating role for osteoclasts. Elevated values of BSP in patients with rheumatoid arthritis and postmenopausal women have been reported [66, 67].

**Cathepsin K:** Cathepsin K is an enzyme from the cysteine protease family. It is predominantly expressed by active osteoclasts. It is a marker of fracture risk and bone mass due to the destruction of the matrix proteins of the bone. There are currently several studies of cathepsin K inhibitors as a potential treatment option for osteoporosis. Although bone resorption is a potentially related marker, further studies are needed for clinical use [68, 69].

#### New bone markers

**Periostin:** Periostin is a protein expressed by collagen-rich connective tissue, including bone. Its role in collagen synthesis is to increase the activation of lysyl oxidase, which allows the collagen to be cross-linked. Periostin has positive effects on both bone formation and bone strength. It has been con-

sidered that periostin could become a marker to demonstrate the differentiation of the metabolic activity of the periosteum. Study results also supported that periostin may be related to the development of oncogenesis, lung disease, and kidney fibrosis [70, 71].

**RANKL and OPG system:** One of the main regulators of osteoclast differentiation and function is the RANKL/RANK/OPG system [20, 22]. It has been suggested that circulating levels of OPG and RANKL could reflect local bone marrow production [72, 73]. The activator of the nuclear factor kappa-B ligand is OPG. OPG is a member of the tumor necrosis factor (TNF) superfamily and inhibits osteoclasts both in formation and activity (Fig. 1). RANKL is a member of the TNF receptor superfamily and is produced by osteoid and preosteoblasts as well as endothelial cells. RANKL binds to the RANK receptor and increases osteoclast formation and activity. Osteoclast precursors express RANK. There are conflicting data on the relationship between these systems and bone disorders [74-77]. The inconsistencies in the results indicate that there is a need for further development of the test methods and better understanding of preanalytical variability.

**Sclerostin:** Sclerostin is a 22 kDa glycoprotein produced by the *SOST* gene. It is expressed in osteocytes and is an antagonist to Wnt signaling [29, 77-79]. Sclerostin reduces bone formation as well as bone resorption. It has been reported that monoclonal antibodies against sclerostin decrease bone resorption and that sclerostin could be accepted as potential biomarker of bone formation [80]. Several studies have suggested a relationship between sclerostin and osteoporosis [81, 82]. Elevated sclerostin levels correlated with estradiol and parathyroid hormone (PTH) observed in high levels in postmenopausal women have been reported as having a protective function [78, 80]. Sclerostin levels increase with age in both sexes [83]. Studies in patients with type 2 diabetes, chronic kidney disease (CKD), or rheumatoid arthritis have indicated that sclerostin could be a marker of vascular calcification. Recently, it has been shown that sclerostin was stimulated by TNF- $\alpha$ , and demonstrated a correction of the bone remodeling uncoupling that occurs due to inflammation [84-87]. However, sclerostin assay standardization is needed before sclerostin can be used clinically for the management of bone disease.

**Dickkopf-1 (Dkk-1):** The Wingless signaling pathway plays an important role in the differentiation and activity of osteoblasts. Dkk 1 is one of the proteins that inhibit this pathway [29, 88-91]. Serum Dkk-1 levels were found to be elevated in multiple myeloma, bone metastases, rheumatoid arthritis, CKD, osteodystrophy, and vascular calcification [91-95]. It has been reported that Dkk-1 levels were negatively correlated with bone mineral density in postmenopausal patients [96]. Circulating Dkk-1 does not, however, accurately reflect bone turnover.

**Sphingosine-1-phosphate (S1P):** S1P is a lipid mediator and has several G protein-coupled receptors [97]. These are

the SP1R, SP1R1, and SP1R2 receptors. S1P affects the proliferation, survival, and migration of osteoblasts, as well as osteoclast differentiation by increasing RANKL in osteoblasts. It controls the traffic of osteoclast precursors between the blood and bone marrow cavities. The S1P concentration is high in the circulation, while it is low in bone; therefore, osteoclast precursors migrate from the blood to bone [29, 98, 99]. S1PR1 and S1PR2 have opposite effects. A low S1PR1 level in monocyte cells results in an accumulation of osteoclast precursors and increased bone resorption, while a decreased S1PR2 level is related to a decrease in osteoclastic bone resorption [100, 101]. It has been suggested that elevated serum S1P levels are associated with elevated bone resorption and increased prevalence of vertebral fractures in women after menopause [102].

**Fibroblast growth factor 23 (FGF23):** FGF23 is synthesized by osteocytes as a molecule of 251 amino acids [10, 29]. FGF23 acts locally within the bone and affects phosphate homeostasis in the kidneys. It has been accepted that FGF23 is a true bone-derived hormone. Klotho, as a cofactor, is required for FGF23 to bind to receptors, with the consequent reduction of phosphate reabsorption in the proximal tubules of the kidney. FGF23 regulates 1-alpha-hydroxylase, serum phosphate, and (PTH). A group of proteins, small integrin-binding ligand, N-linked glycoproteins, is thought to be related to the regulation of FGF23 [103-106]. In the circulation, FGF23 is present in 3 forms: the intact form, and the C- and N-terminal fragments. Prospective studies suggest that there may be a relationship between elevated serum intact FGF23 and fracture risk in the elderly. FGF23 levels can also be predictive of cardiovascular events in patients with CKD, obesity, insulin resistance, and cardiovascular disease [106-111].

**MicroRNAs (MiRNAs):** Single-stranded RNA molecules with 18 to 24 nucleotides, MiRNAs play a regulatory function in several pathways, including organogenesis, cell apoptosis, proliferation, and differentiation [29, 112]. They may be free or bound to proteins. Circulating miRNAs act as signaling molecules that affect epigenetic information between cells. Several studies have examined the role of miRNAs in bone turnover and bone-associated MiRNAs have been identified [112-115]. For example, while miR-34a is target of osteoclastogenesis, miR-133a is relatively specific to the regulation of bone formation [116-120]. Unfortunately, the roles of MiRNAs are very complicated and further studies are needed.

#### Conditions and factors that should be considered and standardized when evaluating bone turnover markers

The ideal BTM should be bone-specific, demonstrate the risk of fracture, provide an assessment of the efficacy of treatment, have a standardized method of analysis and low biological variability. In addition, easy and reliable collection of the sample and analysis that is suitable for automation are other important features of the ideal bone marker [28-32]. It is expected that a BTM will reflect precise changes in bone turnover, and that urine or serum marker levels will be high in cases of increased bone turnover and low in decreased bone turnover (Table 4).

Each of the markers described above has several advantages and disadvantages [29]. The total testing process begins with an order for the test by the presiding physician. This is followed by preanalytical, analytical, and postanalytical phases. Care must be taken at each stage to obtain and interpret the results correctly.

**Table 4. Interpretation of bone turnover markers**

Clinical status	Interpretation of bone turnover markers
Hyperthyroidism	s-CTX ↑
Hyperparathyroidism	u-NTX ↑
Postmenopausal women, Paget disease or bone metastasis	Most marker levels ↑; u- NTX excretion and s-BSAP and s-PINP are very sensitive Serum osteocalcin levels may be in normal range
Antiresorptive therapy	Most bone marker levels ↓ during antiresorptive therapy, depending on treatment and bone marker
Osteoporosis	s-PINP and s-CTX ↑
Fracture	Most bone markers ↑ after a fracture, maximal at 2-12 weeks, but effect lasts for up to 1 year
Fracture risk	The association between bone formation markers and fracture risk was not statistically significant (especially for OC, BALP, PICP and PINP)
Chronic kidney disease	s-OC, s-CTX, s-BSP ↑
Liver diseases	BALP, PICP, PINP, OHP, PYD, DPY, CTX, BSP ↑
Hemolysis	s-TRACP ↑
Drugs	Glucocorticoids reduced bone turnover marker levels Anticonvulsants increased bone turnover marker levels Oral contraceptive reduced bone turnover marker levels with >35 years of use

BALP: Bone-specific alkaline phosphatase; BSP: Bone sialoprotein; CTX: Type 1 collagen carboxy terminal cross-linked telopeptide; DPY: Deoxypyridinoline; NTX: Type 1 collagen amino terminal cross-linked telopeptide; OC: Osteocalcin; OHP: Hydroxyproline; PICP: Procollagen type 1 C-terminal propeptide; PINP: Procollagen type 1 N-terminal propeptide; PYD: Pyridinoline; S: Serum; TRACP: Tartrate-resistant acid phosphatase; U: Urine.

**Table 5. Preanalytical factors affecting bone turnover markers**

	Uncontrolled	Controlled
Less important	Oral contraceptive use	Seasonal rhythm Menstruation Diet
Important	Ethnicity Geography Immobility Diseases Drugs Pregnancy and lactation	Exercises Fasting status
Very important	Age	Circadian rhythm

In the next section, preanalytical factors and factors interfering with bone markers are discussed (Table 5, 6).

**Circadian rhythm:** Circadian rhythm and nutrition affect the level of many bone markers in the blood and urine [122-124]. Knowing and accounting for this is important, and impacts the time of day when test samples are to be taken. Many bone markers have a diurnal variation. TRAP-5b and BALP values seem less affected by the time of day. BTM concentrations peak in the early morning and decrease in the afternoon and evening. CTX has the highest amplitude (60%) in circadian variation. The fact that circadian variability is very low and not affected by food intake is important for the clinical usefulness of PINP. The concentration of urinary bone markers can vary 20% to 30%. Generally, although the within-day and between-day variability of urine markers is similar, the diurnal variability of serum levels of bone formation markers is less than that of between-day variability. BTMs concentrations in urine should be expressed as the ratio of urinary creatinine concentrations. Additionally, bone turnover is higher in the winter months [47]. Bone-building markers are thought to be less affected by fasting and circadian rhythm. Serum samples for CTX measurement should be taken in the morning after overnight fasting. OHP levels are affected by dietary intake. Also, calcium intake leads to a reduction in the concentration of bone resorption markers. Therefore, taking samples after morning and night fasting at between 8 and 10 AM is important for standardization. [123, 124].

**Menstrual cycle:** In premenopausal women, the rate of bone turnover is altered during the menstrual cycle; however, the change is usually not significant. The rate of bone turnover rises in the late follicular phase of the menstrual cycle and falls through the midluteal phase. Therefore, it is most appropriate to take test samples in the follicular phase of the cycle [22].

**Sample stability:** Serum, ethylenediaminetetraacetic acid (EDTA), or heparin plasma may be used for the collection of blood samples. EDTA plasma is advised for the assay of the majority of BTM, but the evidence remains weak. EDTA plasma is not used for calcium or alkaline phosphatase analysis. EDTA is preferred for CTX-1 for plasma sample stability. The storage

conditions are particularly important for serum OC and TRACP. Although long-term storage below  $-20^{\circ}\text{C}$  is suitable for most BTMs, storage conditions below  $-80^{\circ}\text{C}$  are not recommended for OC and TRACP. Repeated freezing and thawing of samples disrupts the analyte quality, and therefore, the analysis of BTMs in such samples is not appropriate [22, 125].

**Exercise:** Exercise leads to both acute and chronic changes in the level of bone markers. The effect depends on age and the type of exercise. Exercise and physical activity may be related to reduced bone turnover. It is recommended that samples should be taken at least 48 hours after exercise [22, 126, 127].

**Age and Sex:** Age is an uncontrolled preanalytical factor in bone marker tests. Children have a significantly higher bone turnover than adults. Therefore, the BTM level is higher in children than in adults [128]. As bone formation accelerates in puberty, bone formation markers increase. In women, there are periodic changes in bone metabolism both after pregnancy and menopause. BTM levels increase during pregnancy, with the highest level reached in the sixth month. Increases in the level of BTMs may continue after delivery. Both resorption and formation markers were elevated in post-menopausal women when hormone replacement therapy was not taken. An increase in BMT level also occurs within a few months after the last period. Bone turnover increases with aging due to dietary calcium deficiency and/or vitamin D deficiency in women [22, 129-131].

Serum and urine BTM concentrations are relatively high in men, with some aging-associated decrease. It has been suggested that the reference intervals of PINP and CTX for men be reduced according to age. BAP has not been seen to significantly change with age in men. The level of BTMs is greater in older women than older men. Until the age of 60 to 70 years, the BTM level remains largely stable in men. Thereafter, bone resorption markers may increase or remain the same, while markers of bone formation may decrease, increase, or remain unchanged [60, 129, 131].

**Drugs:** BTMs are used increasingly to treat patients with metabolic bone diseases, such as Paget's disease, osteoporosis, and metastasized bone cancer. Since anti-resorptive drugs inhibit bone resorption, these markers gradually decline and eventually reach a plateau. Since bone formation continues, the markers of bone formation may remain stable for several weeks, then progressively decrease and reach a plateau. Studies indicate that different responses may be seen in BTM levels according to the mechanism of action and the type of drug as well as the route of administration [39, 132-134].

**Kidney diseases:** In CKD, bone metabolism is often affected by complex and multifactorial mechanisms. [135, 136]. Changes in bone metabolism are largely related to PTH secretion. The role of PTH is the regulation of calcium metabolism. PTH is not an ideal bone marker. In addition, problems of measurement have still not been eliminated, and the biological variability is also quite high. BALP is the bone biomarker measurement recommended by guidelines for renal diseases. BALP is not affected by kidney function. However, there is a need for BALP



**Table 6. Preanalytical factors for bone turnover markers**

Marker	Source	Interpretation
Bone alkaline phosphatase (BALP)	Osteoblast membrane-bound tetramer enzyme	It is bone-specific but can cross-react with liver isoforms up to 10%. The results can be adversely affected by liver alkaline phosphatase level. The effect of circadian rhythm is very low.
Procollagen type 1 N-terminal propeptide (P1NP)	Precursor molecules of collagen type 1 synthesized by osteoblasts	Tests have been developed for intact or total forms. The effect of circadian rhythm is very low. It is the most sensitive marker of bone formation and is particularly useful in monitoring bone formation and anti-resorptive therapies. The biological and analytical variability of serum P1NP has been well documented
Procollagen type 1 C-terminal propeptide (P1CP)	Precursor molecules of collagen type 1 synthesized by osteoblasts	It is mostly derived from bone collagen type 1 (90%) The effect of circadian rhythm is very low.
Type 1 collagen amino terminal cross-linked telopeptide (NTX) Type 1 collagen carboxy terminal cross-linked telopeptide (CTX)	Serum NTX formed by osteoclastic hydrolysis of type 1 collagen. Serum CTX is always $\beta$ -isomerized. It is formed by osteoclastic hydrolysis of collagen. Cathepsin K releases CTX	The effect of circadian rhythm is high. Test samples require prior fasting The level is influenced by kidney and liver functions The effect of circadian rhythm is high. The biological and analytical variability of s-CTX has been well documented
Urinary Deoxypyridinoline (DPY)	Proteolytic hydrolysis of collagen	EDTA is preferred for plasma samples Present in mature collagen only It is independent of dietary intake
Urinary pyridinoline (PYD)	Bone, cartilage, tendon, blood vessels	It is influenced by UV radiation and circadian rhythm Present in mature collagen only It is independent of dietary intake It is influenced by liver function, active arthritis and UV radiation The effect of circadian is high
Serum tartrate-resistant acid phosphatase	Platelets, erythrocytes osteoclasts	Results should be provided as the ratio of creatinine It is influenced by hemolysis The effect of circadian is high
Serum/urine osteocalcin	Osteoblasts and odontoblasts;	Sample stability: 2 years at $-80^{\circ}\text{C}$ Osteoblast function marker It is influenced by the kidneys The effect of circadian is high

measurement standardization in terms of reference intervals and analysis methods [127, 39, 135]. In some studies, it has been suggested that an increase in BALP, PINP and TRAP5b levels in CDK patients indicates a fracture risk. It has been reported that BALP and RAP5b can be used to evaluate bone loss in dialysis patients. Since these 2 markers are not affected by renal clearance and have low biological variability, their clinical utility may be high [136]. Furthermore, recent studies suggest that FGF23 may also be useful in assessing bone metabolism associated with CKD [108, 137, 138].

## Conclusion

BTMs have been measured in serum or urine in a number of clinical studies for use in the diagnosis and follow-up of primary or secondary diseases of the bone. Most of the research has indicated that limited or incompatible results were related to problems in the analysis methods of the markers and the effects of preanalytical variations. In addition, for many BTMs, age- and sex-related reference ranges and clinically specific cutoff values were not identified. Bone markers cannot be used in the evalua-

tion of bone mass and structure, in the amount of bone formed or resorbed, or the diagnosis of bone diseases. Bone markers reflect acute changes in bone metabolism and display all metabolic activity in the skeletal system for a certain time interval.

- Majority of the turnover markers reflect total skeletal turnover and are not always specific to bone metabolism.
- BTMs are valuable for monitoring bone metabolism in patients with CKD. BALP, P1NP, TRAP5a, and FGF23 are biomarkers for follow-up in CKD.
- BALP and P1NP are recommended for both diagnosis and follow-up of Paget's disease.
- BALP, P1NP, and CTX are useful BTMs for the management of primary osteoporosis. The International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine Working Group suggests that CTX may be a useful marker for bone resorption and P1NP for bone formation.
- BALP in tumor-induced osteomalacia can be useful as a follow-up and a diagnostic marker, and FGF23 can be helpful in cases of hypophosphatemic rickets.
- The response of BTMs to anti-adsorptive treatment may be quite different depending on the type of drug, the route of administration, and the characteristics of the disease.
- There are different methods for the same analyte. Therefore, it is important to carry out serial measurements in the same laboratory and using the same method.
- The concentration of BTMs in urine should be expressed as a ratio of urinary creatinine concentration.
- For plasma samples, EDTA or heparin is generally suitable as an anticoagulant. Hemolyzed serum samples should not be used for enzyme analysis, particularly for TRAP.
- Measurement of BTMs (especially for beta CTX-1 and P1NP) after fasting is currently recommended.

In spite of the large number of studies, the role of bone markers in clinical guidelines remains very limited. The majority of markers are specific to collagen formation and destruction. Variables such as age, gender, and menopause are important because of the dynamic nature of bone as well as lifelong modeling and remodeling activities. The fact that most of the markers are peptide or protein in structure and are easily hydrolyzed in plasma affects the sensitivity and specificity of the analysis method. It is also important that the method is suitable for automation. Unfortunately, at present, automated analysis is available for only a few bone markers. Bone mineral density measurement remains important; however, measurement of bone markers can be achieved more quickly. Therefore, standardization and harmonization studies of bone markers are very important.

**Conflict of interest:** None declared.

**Financial Disclosure:** None declared.

**Peer-review:** Externally peer-reviewed.

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