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A Glance at Biomarkers of Bone Metabolism

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Abstract: The biomarkers of bone metabolism are proteins, which are synthesized by the osteoblast and osteoclast and are released into the bloodstream or circulation as fragments during the formation or degradation of type I collagen. Because some of these fragments are small, they are filtered by the kidney into urine, but the larger fragments which are not filtered are detected in the blood samples. Disorders or disease conditions of skeletal metabolism result in the release of these fragments, which are products of osteoblastic and/or osteoclastic activity. Measurements of these biomarkers of bone metabolism by immunoassay methods show promise as important adjuncts in the early diagnosis of bone changes. These biomarkers are also important in the evaluation and management of skeletal complications of sickle cell disease and other disorders of bone; however they are affected by a variety of physiological and pathological factors. In conclusion, biochemical bone biomarkers are convenient, non-invasive indices of bone metabolic activities, but other analytes such as parathyroid hormone and 25-hydroxycholecalciferol or calcidiol may also be considered.

Key words: Alkaline phosphatase, osteocalcin, procollagen I peptides, pyridinoline crosslinks, telopeptides

INTRODUCTION

Bone is a living and dynamic tissue (Bolarin, 2013). Bone remodeling occurs throughout the life of an individual. Throughout life, bone is renewed by the process of bone formation and bone resorption (Thomas, 2012). The products of these physiological changes in bone are known as bone turnover biomarkers (Thomas, 2012). The rate of bone formation or bone degradation or resorption can be assessed by:

a: Measurement or estimation of a prominent enzymatic activity of the bone forming cells or bone resorbing cells
b: Measurement or estimation of bone matrix components secreted or released into the bloodstream or circulation during bone formation or bone degradation or resorption

These bone turnover biomarkers are classified as bone formation biomarkers and bone resorption or degradation biomarkers. The bone biomarkers are applied as quantitative and dynamic indication of current bone changes, but bone density estimations or measurements reveal or indicate events that affected bone turnover over preceding months to years (Thomas, 2012; Fontana and Delmas, 2000). When both bone formation and bone resorption or degradation are linked and in balance either of the biomarkers will reflect the overall rate of bone turnover (Thomas, 2012; Fontana and Delmas, 2000; Bolarin, 2005).

Remodeling cycle of bone: The bone remodeling cycle starts with the mobilization of osteoclast precursor cells, which then differentiate into osteoclasts, when they receive signals from the osteoblasts (Thomas, 2012; Bolarin, 2005). The matured osteoclasts synthesize and release lysosomal enzymes, which are necessary for bone resorption by digesting the collagen matrix. Bone resorption is the first phase of the remodeling cycle. The length of the first phase is determined by apoptosis of osteoclasts (Thomas, 2012; Bolarin, 2005). The next phase of the remodeling cycle is the attraction of pre-osteoblasts from mesenchymal stem cells in the bone marrow (Thomas, 2012). The mesenchymal stem cells are giant multinucleated primitive cells called the osteoprogenitor cells. Some hormones and some bone proteins known as skeletal growth factors accelerate the differentiation of osteoprogenitor cells into osteoblasts (Bolarin, 2005). The growth of the osteoblasts is also stimulated by the growth factors (Thomas, 2012; Bolarin, 2005; Garnero *et al*., 2000). The matured osteoblasts synthesize the bone matrix, which is mainly type I collagen and the osteoblasts also regulate the mineralization of the newly formed bone (Garnero *et al*., 2000; Sorva *et al*., 1996; Akesson, 1995; Bolarin, 1996; Bolarin, 2013). The osteoblasts are rich in enzymes,

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especially alkaline phosphatase, which is important for the deposition of calcium in the bone matrix (i.e., calcification). Osteoblasts also produce gla-protein and osteopontin that are necessary for calcification. After bone formation the osteoblasts differentiate into osteocytes that are trapped inside the lacunae of calcified bone (Bolarin, 2005). It is expected that an ideal biomarker of bone metabolism should posses the following properties: It should be specific for bone and for one of its metabolic process, have high disease sensitivity, it must have a long half-life in the serum or plasma since a marker with very short half-life (such as few minutes) is not ideal to measure, its blood clearance method is very important (since impaired kidney function may result in an elevated level of a marker that is cleared by kidney), where a biochemical marker is metabolized and its fragments are released into the blood circulation, then an assay that measures not only the intact molecules of the marker but also fragments will give false results and, When a urinary biomarker is measured, in order to be ideal as biomarker, it should be cleared completely from bloodstream by the kidneys and not metabolized (Bolarin, 2005; Sorva *et al*., 1996; Kleerekoper, 1997).

Biomarkers of bone formation: Table 1 gives a summary of some of the characteristics of the biomarkers of bone formation currently in use in most clinical laboratories.

Bone formation biomarkers are the products of the osteoblasts and their activity. And they include:

a) **Propeptides of type I procollagen:** Type I collagen is the major structural or connective tissue protein of the bone matrix. Type I collagen is synthesized as a large precursor called type I procollagen. This then undergoes proteolytic cleavage. The amino-terminal and carboxy-terminal ends of the procollagen molecule are cleaved off by specific peptidases, once the procollagen has been secreted into the extracellular fluid. This cleavage must be completed to ensure correct fibril and fibre formation (Kleerekoper, 1997). The peptide cleavage from carboxy-terminal end of the procollagen molecule is known as the carboxy-terminal propeptide of type I procollagen (PICP), While the peptide cleaved from the amino-terminal end of the procollagen molecule is called amino-terminal propeptide of type I procollagen (PINP) and these are released into the bloodstream. The depositions of new type I collagen in bone can be measured on a one to one stoichiometrically by estimation of the either PICP or PINP in the blood (Akesson, 1995; Bolarin, 1996; Kleerekoper, 1997; Bolarin, 1986). The levels of either PICP or PINP in plasma or serum reflect the rate of bone formation (Garnero *et al*., 2000; Sorva *et al*., 1996; Akesson, 1995; Bolarin, 1986). Data from our study in sickle cell haemoglobinopathy suggested a relationship between an early rise in PICP and skeletal changes (Bolarin, 2001; Bolarin *et al*., 1998).

b) **Alkaline phosphatase:** The total serum alkaline phosphatase consists of several isoenzymes. These isoenzymes are from bone, liver, intestine, spleen, kidney and placenta. About 50% of the total serum alkaline phosphatase activity in healthy adult subjects is from the liver (i.e., hepatic origin), but the rest is from the bone (i.e., bone origin). Bone-specific alkaline phosphatase is synthesized and released by the osteoblasts. The bone-specific alkaline phosphatase reflects the activity of the osteoblast during bone formation. The enzyme activity correlates with bone formation rate in metabolic disease of the bone (Akesson, 1995; Bolarin, 1996; Bolarin, 2001) and during normal physiological bone growth (Akesson, 1995; Bolarin, 1996; Bolarin, 2001).

   There are various techniques for the measurement of bone-specific alkaline phosphatase. These methods include physical and chemical techniques, which are used to differentiate liver and bone isoenzymes in serum samples. In the absence of hepatic disorders and with other hepatic enzymes within normal reference ranges, an elevated total alkaline phosphatase is considered to represent an increase in bone-specific alkaline phosphatase. An immunoradiometric assay and other immunoassays that preferentially recognize bone-specific alkaline phosphatase are generally being used in most laboratories (Bolarin, 2005; Garnero *et al*., 2000; Akesson, 1995; Bolarin, 1996; Bolarin, 1986). These assay methods have shown improved diagnostic accuracy with regards to bone diseases and their therapeutic monitoring. Serum bone-specific alkaline phosphatase is not routinely determined in our laboratories because of the cost to the patient Bolarin, 1996, 2001).

   The serum level of bone-specific alkaline phosphatase is significantly associated with fracture risk regardless of bone mineral density in postmenopausal females (Bolarin, 2005; Garnero *et al*., 2000). The bone-specific enzyme can be applied to monitor progress in Paget's disease and other bone disorders (Bolarin, 2001). It is a sensitive biomarker of bone turnover and could be especially useful as a valuable non-invasive biomarker for identifying sickle cell patients with skeletal complications (Bolarin, 1986; Bolarin and Azinge, 2010).

c) **Osteocalcin:** Recent discovery is the unexpected functions played by the skeleton in whole-organism

physiology. Among these newly described functions is the role of bone in the control of energy metabolism, which is achieved through the release of osteocalcin, an osteoblasts-derived hormone regulating insulin secretion, insulin sensitivity and energy expenditure (Ferron *et al*., 2008; Hwang *et al*., 2009). Osteocalcin or bone gamma-carboxyglutamic acid (gla) protein is the most abundant non-collagenous protein found in bone (Akesson, 1995; Bolarin, 1996; Bolarin, 2001).

Osteocalcin is a vitamin K-dependent, calcium binding protein Akesson, 1995; Bolarin, 1996; Bolarin, 2001). Most of the osteocalcin that is synthesized by the osteoblasts is incorporated into or binds to hydroxyapatite in the bone matrix and the rest is released into the bloodstream. Osteocalcin represents the activity of the osteoblasts. In addition to osteocalcin regulating bone remodeling through a negative feedback mechanism, osteocalcin is also an endocrine factor regulating glucose homeostasis as stated above (Akesson, 1995; Bolarin, 1996, 2013; Kleerekoper, 1997; Garnero *et al*., 1994; Bolarin and Azinge, 2010; Ferron *et al*., 2008; Hwang *et al*., 2009). Osteocalcin is unstable hence once collected it must be measured or stored appropriately. Osteocalcin fragments have been detected in the bloodstream and these are due to degradation by circulating proteases; part of these fragments may also be released during bone resorption (Thomas, 2012; Bolarin, 2005; Akesson, 1995; Bolarin, 1996).

Measurement of osteocalcin is by immunoassays using monoclonal or polyclonal antibodies. It is advised that blood samples be collected in ice and the plasma or serum is stored at -20 or -70°C. Sample should be thawed once to prevent degradation of the protein. Because of the heterogeneity of circulating osteocalcin, different assays give differing results (Thomas, 2012; Fontana and Delmas, 2000; Kleerekoper, 1997; Garnero *et al*., 1994; Bolarin and Azinge, 2010; Ferron *et al*., 2008; Hwang *et al*., 2009).

Biomarkers of bone resorption: Table 2 gives a summary of some of the characteristics of the biomarkers of bone resorption or degradation currently in use in most clinical laboratories.

Biomarkers of bone resorption or degradation are mainly type I collagen degradation products. These products indicate the degree of bone matrix breakdown and indirectly or in some way the number of active osteoclasts (Bolarin, 2005, 1996; Akesson, 1995).

a) **Hydroxyproline:** About 14 per cent of the amino acids content of collagen is hydroxyproline (Thomas, 2012; Bolarin, 2005, 1996; Akesson, 1995). It is a posttranslational modified amino acid or imino acid. It is modified from some prolyl residues in the procollagen by the action of the intracellular enzyme prolyl hydroxylase. Urinary excretion rate of hydroxyproline was used earlier to assess bone resorption rate, however a significant amount of urinary hydroxyproline originates from the degradation of collagen-like structures such as the complement component C1q, acetylcholinesterase and lung surfactant (Bolarin, 2005, 1996; Akesson, 1995) thus the assay has been superseded by more specific assays (Bolarin, 2005, 1996; Akesson, 1995).

b) **Pyridinoline crosslinks:** Urinary pyridinoline and deoxypyridinoline occur in collagens as hydroxylysyl pyridinoline and deoxypyridinoline non-reducible covalent cross-links based on aldehyde forms of hydroxylysines and lysines. They are small, cyclic amino structures linking peptide chains of collagen molecules. These interconnections of the collagen molecule increase tensile strength of the collagen. These cross-links are formed in the extracellular media after the collagen molecules have been deposited in the matrix. During resorption or degradation these cross-links are released into the bloodstream or circulation (Bolarin, 2005, 1996; Akesson, 1995; Kleerekoper, 1997; Garnero *et al*., 1994; Bolarin and Azinge, 2010; Ferron *et al*., 2008; Hwang *et al*., 2009). These small cross-links can be detected in urine, but about 40% are bound to various proteins. The urinary level of pyridinoline and deoxypyridinoline indicate the rate of collagen degradation or resorption. They are now available immunoassay methods for both urine and serum biomarkers and the immunoassay has been used by one of the authors to measure urinary deoxypyridinoline in patients with sickle cell haemoglobinopathy. The results showed that there is high bone resorption or degradation in the patients with this disorder (Garnero *et al*., 1994; Bolarin and Azinge, 2010). The levels are not affected by diet, but are affected by diurnal variation. It is recommended that sample be taken early in the morning or a 24 h urine collection should be used (Thomas, 2012; Bolarin, 1996; Bolarin and Azinge, 2010; Hwang *et al*., 2009).

c) **Telopeptides**

Cross-links of amino- and carboxy-terminal telopeptides of type I collagen: Collagen molecules in collagen fibres continue to undergo chemical modifications under normal physiological conditions. Intermolecular cross-links are generally formed between the terminals, non-helical telopeptides region one type I collagen molecules and the helical

region of an adjacent collagen molecules by the pyridinoline (Thomas, 2012; Bolarin, 2005; Bolarin, 1996; Garnero *et al*., 1994; Bolarin and Azinge, 2010). They have specific amino acid sequences, which are derived from bone collagen resorption or degradation. The carboxy-terminal telopeptide of type I collagen (ICTP or CTX) and the amino-terminal telopeptide of type I collagen (INTP or NTX) are released into the bloodstream or circulation when bone collagen is degraded during bone remodeling and can be detected in circulation (Thomas, 2012; Bolarin, 2005, 1996; Bolarin and Azinge, 2010). The amino-terminal telopeptides can be estimated or determined in serum, but serum levels of the carboxy-terminal telopeptide of mature collagen are more useful in monitoring progress in osteoporosis and in bone resorption of multiple myeloma (Thomas, 2012; Fontana and Delmas, 2000; De la Piedra *et al*., 1996; Kleerekoper, 1997). A high level has been correlated or linked with an increased risk of fractures independent of bone mineral density (Thomas, 2012; Fontana and Delmas, 2000; De la Piedra *et al*., 1996; Kleerekoper, 1997). Determination of these biomarkers may also be useful in monitoring the response to anti-resorptive therapy or drugs for example, bisphosphonates (De la Piedra *et al*., 1996; Ferron *et al*., 2008; Bonnick and Shulman, 2006; Vasikaran *et al*., 2011; Lipton *et al*., 2011).

The serum assays of carboxy-terminal telopeptides are affected by marked diurnal variation and food. High levels of are seen in menopause. Early morning fasting blood sample is advised.

Factors affecting assay results: Bone turnover biomarkers are physiologically produced and secreted into the bloodstream or circulation during normal bone remodeling cycle. The blood or plasma levels of these biomarkers will be elevated or increased in metabolic bone disorders or diseases (e.g., osteoporosis), other pathological states and during physiological processes, for example fracture healing and skeletal growth (Vasikaran *et al*., 2011; Lipton *et al*., 2011; Coleman *et al*., 2011). These biomarkers are of unequal specificity and sensitivity and none of these biomarkers is disease specific (Thomas, 2012; Fontana and Delmas, 2000; Bolarin, 2005; Akesson, 1995). The biomarkers of bone metabolism may be used by the orthopaedic surgeons or the family doctors to monitor therapy response and disease progression in several bone metabolic disorders or diseases including postmenopausal osteoporosis, hormone-replacement therapy- (HRT-) induced osteoporosis or bone skeletal complications and Paget's disease (Fontana and Delmas, 2000; De la Piedra *et al*., 1996; Kleerekoper, 1997; Bonnick and Shulman, 2006; Vasikaran *et al*., 2011). Frequent complications of cancer disease are bone metastases. The assessment of metastatic bone disease by the oncologists is essential for the primary cancer staging as this will affect the therapeutic decision (Fontana and Delmas, 2000). The diagnosis of bone metastases more often than not relies on skeletal imaging, for example, X-ray and bone scintigraphy. The imaging technique or the bone scintigraphy technique is a sensitive diagnostic tool but lacks specificity. Furthermore, the evaluation of effectiveness in the treatment of bone metastasis is difficult because the high radionucleotide uptake does not always indicate an active metastatic area but may relate to a bone reconstruction in patients responding to treatment (Fontana and Delmas, 2000; Lipton *et al*., 2011; Coleman *et al*., 2011). Several factors influence the level of bone turnover biomarkers in the bloodstream and urine. These factors include age, sex, fasting, circadian rhythms, menstrual cycle, exercise history and clinical history.

Result interpretation is best or optimized by taking a thorough or meticulous clinical history and also the collection of samples must be done under standard or normal conditions (Thomas, 2012; Fontana and Delmas, 2000; Garnero *et al*., 2000; Sorva *et al*., 1996). The age of the subject exerts the greatest effect on turnover biomarkers. Children and adolescents have higher levels than adults; hence the first author had advocated the use bone biomarkers in age determination (Thomas, 2012; Fontana and Delmas, 2000; Akesson, 1995; Bolarin, 1996). There are also significant increases in bone biomarkers during skeletal growth (Akesson, 1995; Bolarin, 1996). Sex difference is also seen in that bone turnover biomarkers in females reach a plateau between 20 and 25-year old, while in males it is between 25 and 30 years old, which indicates or suggests peak bone mass (Thomas, 2012; Fontana and Delmas, 2000; Akesson, 1995; Bolarin, 1996). In postmenopausal period the bone biomarkers increase markedly due to reduction on oestrogen and then gradually decline but do return to the premenopausal concentrations. But in males this differs, because bone biomarkers decreases with ageing (Thomas, 2012; Fontana and Delmas, 2000; Akesson, 1995). Food intake has an effect on bone turnover. Dietary calcium tends to inhibit bone resorption or degradation (Bolarin, 2013; Thomas, 2012; Fontana and Delmas, 2000; Akesson, 1995; Bolarin, 1996). Calcium supplement when taken in the evening considerably lower the resorption or degradation biomarkers, in the fasting state, the next morning (Bolarin, 2013; Thomas, 2012; Fontana and Delmas, 2000; Akesson, 1995; Bonnick and Shulman, 2006).

Bone biomarkers have a diurnal rhythm, because they peak in the morning. There have been reports of seasonal variation. Exercise affects bone biomarkers

Table 1: Biomarkers of bone formation (Osteoblastic biomarkers)

Osteoblastic biomarkers	Sample or specimen	Assay technique or method
Bone-specific alkaline phosphatase	Plasma or serum	Enzyme-linked immunosorbent assay (ELISA)
Carboxyterminal propeptide of type I procollagen (PICP)	Plasma or serum	Enzyme-linked immunosorbent assay (ELISA)
Osteocalcin	Plasma or serum	Enzyme-linked immunosorbent assay (ELISA)

Table 2: Biomarkers of bone resorption or degradation (Osteoclastic biomarkers)

Osteoclastic biomarkers	Sample or specimen	Assay technique or method
Pyridinoline (free or total)	Plasma or serum or urine	Enzyme-linked immunosorbent assay (ELISA)
Deoxypyridinoline (free or total)	Plasma or serum or urine	Enzyme-linked immunosorbent assay (ELISA)
Carboxyterminal telopeptide of type I collagen (ICTP or CTX)	Plasma or serum or urine	Enzyme-linked immunosorbent assay (ELISA)

and immobility will result in marked elevated levels of biomarkers of bone resorption or degradation (Thomas, 2012; Bolarin, 2005, 1996; Sorva *et al*., 1996; Akesson, 1995).

Biomarkers of bone metabolism in clinical practice: Current evidence suggests that biomarkers of bone metabolism may be useful in some patients with osteoporosis (Thomas, 2012; Fontana and Delmas, 2000; Bolarin, 2005; Garnero *et al*., 2000; Sorva *et al*., 1996; Akesson, 1995), sickle cell Haemoglobinopathies with skeletal complications (De la Piedra *et al*., 1996; Kleerekoper, 1997; Garnero *et al*., 1994; Bonnick and Shulman, 2006), for monitoring the response to anti-resorptive treatment (Thomas, 2012; Bolarin and Azinge, 2010; Compson, 2009; Vasikaran *et al*., 2011). Studies have reported that intravenous and oral bisphosphonates treatment respectively lead to a decrease in bone resorption or degradation biomarkers (Thomas, 2012; Bolarin and Azinge, 2010; Compson, 2009; Bonnick and Shulman, 2006). The low levels of resorption biomarkers are followed by a decline in bone formation biomarkers (Thomas, 2012; Bolarin and Azinge, 2010; Compson, 2009; Bonnick and Shulman, 2006; Vasikaran *et al*., 2011; Lipton *et al*., 2011). An important change in blood levels of bone biomarkers after starting treatment indicates or confirms compliance (Thomas, 2012; Fontana and Delmas, 2000; Coleman *et al*., 2011).

Conclusion: In conclusion, the assays of the biochemical bone biomarkers show considerable promise as convenient, non-invasive indices of systemic rate of bone formation (osteoblastic activity) and degradation or resorption (osteoclastic activity). These biomarkers of bone turnover are dynamic. The biomarkers of bone metabolism are currently being used in clinical practice in some hospitals abroad. Other analytes such as parathyroid hormone and 25-hydroxyvitamin D or calcidiol should also be considered. The bone biomarkers even though they reflect the rate of bone metabolism or turnover, they have limited clinical utility. They cannot predict fracture risk and may not be used as screening assays in routine clinical practice. Their short coming is lack of common reference intervals and standardization of analytical methods.

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