In this issue: Laboratory investigation of vitamin D and bone metabolism markers

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Laboratory aspects and clinical utility of bone turnover markers

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ABSTRACT

With an aging population, there is a marked increase in prevalence of metabolic bone diseases, especially osteoporosis. Perhaps the most dreaded complication of metabolic bone disease, fractures typically impose a huge burden on the ailing body and are associated with high co-morbidity and mortality. The consequent public health and socioeconomic burden warrant timely diagnosis, treatment and follow-up of these disorders. Knowing the limitations of radiological techniques, biochemical markers of bone turnover measurements come handy since the changes in their levels readily reflect bone physiology. Bone biomarkers typically analyzed in high throughput automated routine laboratories are collagen degradation products, reflecting osteoclast activity, and the collagenous or non-collagenous proteins produced by the osteoblasts. Since bone biomarker levels vary considerably due to quite a few endogenous and exogenous pre-analytical factors, knowledge of these limitations is mandatory prior to clinical utilization since these variabilities complicate test result interpretation. Standardization to harmonize different assay methodologies is desired, and the primary aims of the IFCC/IOF bone marker standards working group are also presented. Current literature data advocate bone markers as best used in monitoring anti-osteoporosis therapy

efficacy and compliance, nonetheless, there is abundant data supporting their role in predicting bone loss and fracture risk. Furthermore, they have widespread clinical utility in osteoporosis, renal osteodystrophy, and certain oncological conditions and rheumatic diseases.

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INTRODUCTION

The interplay of the bone cells, namely, the osteoclasts and the osteoblasts is generally termed as bone turnover. Knowingly, the macrophage-lineage derived osteoclasts are destined to carry out bone resorption and the mesenchyme-lineage derived osteoblasts are responsible for bone formation ideally in a coupled fashion (1,2). The imbalance in their functioning ultimately disrupts bone turnover and is characteristically noticed in metabolic bone disease (3). Per se diagnosis, monitoring, disease severity and treatment efficacy is generally challenging due to the silent, symptomless nature of these disorders, usually at onset, and primarily because the radiological features do not promptly reflect changes in bone metabolism. The high morbidity and mortality associated with these conditions mandate diagnostic procedures that would ideally reflect the actual state of the bone. The present narrative mini-review presents the biomarkers characteristic of osteoblast and osteoclast functioning, namely the markers of bone formation and those of bone resorption, respectively. Ideally changes in levels of the biochemical markers of bone turnover coupled with radiological findings and fracture risk assessment in individual patients ideally identify the patient most susceptible to suffer a non-traumatic fracture. Although dual energy x-ray absorptiometry (DEXA) till date is the gold standard methodology to measure bone mineral density (BMD), it is known that decrease in bone mass does not solely account for fracture risk (4,5). Realizing this limitation, the University of Sheffield, UK launched the fracture risk assessment tool (FRAX) in 2008 under the guidance of Professor John A. Kanis (6), where apart from the femur neck BMD 11 other easily assessable risk factors are included. The FRAX algorithms give the 10-year probability of hip fracture and the 10-year probability of a major osteoporotic fracture (clinical spine, forearm, hip or shoulder fracture) (7). Lately, the addition of bone turnover marker levels to the FRAX algorithms has also been advocated (8). Nonetheless, it is realized that bone mass changes measurable by radiographical techniques, including DEXA, are detectable almost a year following change at the cellular level (9).

Apart from mineralization, which is rather a physicochemical affair, bone turnover reflects bone cellular activity and is a dynamic biological process (10). Given the difficulties of assessing dynamic processes at a static interval, bone histomorphometry using tetracycline double-labeling is the gold standard in determining this feature of bone biology (11,12). Although considered the gold standard, bone histomorphometry has its own limitations, including sampling error, invasiveness, costs, and lack of availability at the primary level. As such, due to limitations of both radiographic techniques and bone histomorphometry, measurement of biomarkers readily assessable from blood samples is an attractive alternative to evaluate bone turnover.

Since all metabolic bone diseases usually present with alterations in osteoblast and osteoclast activity, biochemical markers of bone turnover reflecting these activities mirror real time bone turnover. Furthermore, bone biomarker levels also provide an index of disease activity in certain tumorous and rheumatological diseases affecting bone. The present review summarizes the pre-analytic, analytic and post-analytic implications of bone turnover biomarkers, since knowledge of these limitations is mandatory in correct test result interpretation. Furthermore,

the clinical utility of these biomarkers has been summarized in renal osteodystrophy, certain oncological conditions, rheumatic diseases and Paget's Disease of the bone.

MARKERS OF BONE TURNOVER

Bone biomarkers typically analyzed in high throughput automated routine laboratories are collagen degradation products, reflecting osteoclast activity, and collagenous or non-collagenous proteins produced by the osteoblasts (table 1). All these markers can be quantitated well from blood samples, serum being the preferred sample of choice. Although assays for urine examination were developed for quite a few markers, blood sampling generally detours the pre-analytic issues usually involving urine sampling (13). The most commonly used bone resorption and bone formation markers are discussed below.

BIOMARKERS OF BONE RESORPTION

C- and N-terminal telopeptide of type I collagen

During bone degradation, osteoclast derived tartrate-resistant acid phosphatase (TRAP) and cathepsin K breakdown the bone matrix, including the triple helices of the mature type I collagen, to release carboxy- and nitrogen telopeptide containing fragments (CTx and NTx). The assay designed determines specific amino acid sequence of the telopeptide of Type I collagen termed as crosslaps, and those with β-aspartic acid as βCTx (14). Although its counterpart the N-terminal telopeptide (NTx) can also be measured from urine samples, CTX has gained increased popularity as it can be measured from blood samples on automated platforms, and given the increasing body of literature dealing with this biomarker it may perhaps be stated that it has turned out to be the biomarker of choice to examine osteoclastic bone resorption activity (15). CTx and NTx are both cleared by the kidneys, as such its clinical usefulness in CKD is significantly limited.

At the dawn of bone turnover biomarker development tartrate-resistant acid phosphatase, collagen cross-link molecules pyridinoline and deoxypyridinoline, hydroxyproline and bone sialoprotein saw light of day and were examined to measure osteoclast activity (16). All the aforementioned markers have since been superseded by the more sensitive and specific telopeptides of type I collagen, namely the C-terminal telopeptide (CTx).

Table 1 Biochemical markers of bone turnover

Bone formation markers	Bone resorption markers
Osteocalcin	C-Telopeptide of Collagen Cross-links (CTx)
Bone Specific Alkaline Phosphatase (BSAP)	N-Telopeptide of Collagen Cross-links (NTx)
Carboxyterminal propeptide of Type I Collagen (P1CP)	Pyridinolines
Aminoterminal propeptide of Type I Collagen (P1NP)	Deoxypyridinoline
	Tartrate-Resistant Acid Phosphatase (TRAP)

BIOMARKERS OF BONE FORMATION

N- and C-terminal propeptides of type I collagen

Osteoblasts secrete type I collagen as a procollagen which forms a triple helix (containing two α- and β-chain), and contains the N- and C-terminal propeptides (P1NP and P1CP), these propeptides are immediately cleaved in its extracellular vicinity eventually entering the blood circulation (17). As such, the N- and C-terminal propeptides qualify themselves as being biochemical markers of bone formation (18). The cleaved products are initially in the trimeric form that are eventually broken down to the monomeric form in the circulation the trimeric P1NP is cleared by hepatic uptake, while the monomeric form is cleared via the kidneys. Assays measure the monomeric and trimeric forms (total P1NP) or only the trimeric form (intact P1NP) (19). Being dependent on renal clearence, the monomeric form of P1NP accumulates in chronic kidney disease (20,21).

Osteocalcin

Osteocalcin is the most abundant non-collagenous protein in the bone composed of 49 amino acids and is secreted by the mature bone formation cells the osteoblasts. It is also known as bone gamma-carboxyglutamic acid-containing protein since it contains 3 glutamic acids at positions 13, 17 and 20 that undergo gamma-carboxylation in a vitamin-K dependent fashion (22). It is noteworthy here that patients on vitamin-K antagonists (e.g., warfarin) show decreased osteocalcin concentrations. Osteocalcin has appeared again recently in the reflector light, following the identification of its role as a bone derived hormone influencing male fertility, glucose metabolism, and its actions on the central nervous system and muscle in animal experiments (23-25). Although primarily identified as a marker of bone formation, due to its tight correlation with bone formation measurements by bone histomorphometry, it may well be considered a marker reflecting both formation and resorption, i.e, a marker of bone turnover since it is also liberated during osteoclastic bone resorption (19, 26).

Due to its labile 6-amino acid C-terminal sequence samples for osteocalcin determination have traditionally required special collection and transportation requirements, this has been overcome by development of assays that determine the more stable N-MID fragment (27,28). Nonetheless, osteocalcin measurement has limited value in patients with reduced renal function since its mainly cleared by the kidneys (29).

Bone-specific alkaline phosphatase

In the healthy adult, almost half of the circulation total alkaline phosphatase is derived from the bone, i.e., produced by the osteoblasts and the remainder is constituted by the fraction produced by the hepatocytes (30,31). Bone-specific alkaline phosphatase (BSAP) primarily inactivates the mineralization inhibitor pyrophosphate (32). Although commercial assays are available to measure BSAP, they show cross-reactivity with liver alkaline phosphate, as such in patients with liver disease BSAP measurements have limited applicability (19). BSAP show good correlation with fracture risk in CKD populations (33). Although a disease of the osteoclasts, it has been reported that BSAP proved to be sensitive in monitoring disease progress in patients suffering from Paget's disease (34).

In summary, as compared to the bone resorption biomarkers, there is a larger repertoire of biomarkers of bone formation, reflecting osteoblast activity, that can be used in automated high throughput laboratories, namely serum bone-specific alkaline phosphatase, osteocalcin and procollagen type I N-terminal propeptide (PINP). Although produced by the osteoblasts,

osteocalcin may be defined as a bone turnover marker reflecting both bone formation and bone resorption, since it is also released from the bone matrix during bone resorption. P1NP is more extensively described in literature are compared to the other bone formation biomarkers (35).

PRE-ANALYTICAL AND ANALYTICAL CONSIDERATIONS IN ROUTINE LABORATORY DETERMINATION OF BIOMARKERS OF BONE TURNOVER

Biomarkers of bone turnover are quite sensitive to a number of pre-analytical and analytical issues.

Technical pre-analytical issues pertaining to sample collection are implicated mainly in urinary sample collection (13). As mentioned earlier, due to the cumbersomeness of spot or 24 hr urine sample collection and the need for correction for creatinine, urine sampling has started to go out of fashion and blood sampling is the preferred mode of sample collection.

Perhaps the major challenge is the over-coming of biological factors that cause variability in test results. Although trivial for the professional at home with the markers of bone turnover, one needs to be reminded of a number of endogenous and exogenous factors that should be mandatorily considered before interpreting test results.

Normal blood levels of bone turnover markers is usually higher in children, depending on the biomarker these elevations may well be a multifold of those expected in the adult population (16,36). The levels in the elderly usually show a decline, but there usually is an elevation in women following menopause (16,37,38). Levels are albeit usually higher in men as compared to women (16,38). The ethnic background of the patient is also to be considered since data suggest that the Caucasians usually have lower levels as compared to their age and sex matched adult counterparts (16,39).

Marker levels are elevated during pregnancy and lactation and tend to normalize after a few months following weaning (40,41). Marked elevations have been reported in marker levels in those immobilized or bedridden for any reason (42). Marker levels may be significantly elevated even at 6 months following a bone fracture (43-45). Patient with concomitant comorbidities such as primary hyperparathyroidism, Paget's disease, multiple myeloma and metastatic prostate and breast cancer usual present with higher levels (46-52). Abnormal kidney function results in elevated marker levels, particularly monomeric P1NP, CTx and osteocalcin, these mainly undergo renal clearance (53).

Biomarker levels vary considerable due to quite a few endogenous factors, as such one needs to take into consideration the circadian rhythm, the phase of the menstrual period, seasonal variation, physical exercise and diet.

Bone markers, particularly the resorption markers, follow a circadian rhythm where the peak levels are typically observed in the early morning hours and the levels tapper off during the day (16,37).

Biomarkers of bone formation are characteristically elevated following ovulation, i.e., during the luteal phase (54). On the other hand, resorption markers are elevated during the follicular phase of the menstrual period (55). Biomarker levels of both resorption and formation reflect vitamin D status in the winter months; this usually translates into these levels being higher during this time of the year (56). Given the literature till date, there is no clear consensus on the effect of exercise on bone turnover biomarker levels (57,58). A meat or gelatin rich diet usual results in elevated marker levels (59). Patients may be advised to have an overnight fast before

the examination. High basal bone biomarkers levels are usually observed in current smokers and those with low body mass index (60). Since most bone turnover markers are also present in other non-skeletal organs with type I collagen, cardiac conditions and systemic sclerosis, e.g., have also shown to present with elevated bone biomarker concentrations (61-63).

There is marked analytical variability of bone biomarkers. The methodology is not standardized and using different assays from various manufactures generally present a huge difference in test results from the same sample. Task forces or working groups like the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) bone marker standards in affiliation with the International Osteoporosis Foundation (IOF) are working arduously to harmonize various assay methodologies (64-66). Additionally, participation in external quality control schemes could help minimize inter-laboratory variations (15).

IFCC WORKING GROUP ON STANDARDISATION OF BONE MARKER ASSAYS (WG-SBMA) IN COLLABORATION WITH IOF

The WG-SBMA is currently headed by Professor Etienne Cavalier, and the following are the terms of reference and projects for the period 2018-2020, as presented in the *IFCC Handbook 2018-2020* (67).

Terms of reference

- To standardise or harmonise clinical assays available for routine and research use, for the following two bone turnover markers; the serum assay for CTx and P1NP.

Current projects

- Review literature and current status of available assays in order to develop and undertake a project to establish a reference measurement system for serum β-CTx or harmonisation of the assays for serum β-CTx as appropriate.
- Review literature and current status of available assays in order to develop and undertake a project to establish a reference measurement system for serum P1NP or harmonisation of the assays for serum P1NP as appropriate.
- Review and identify data required for the regulatory authorisation of these modified assays.
- Review literature and consider the critical decision limits and potential target levels of serum β-CTx and serum P1NP for treatment of postmenopausal osteoporosis and other causes of osteoporosis as appropriate
- IOF-IFCC study to summarize fracture prediction strength of reference bone turnover markers.

The above is in line with their position paper published in 2001, where they advocate use of P1NP and CTx (i.e., one formation and one resorption marker) in clinical trials and other studies to improve our understanding of bone biomarkers and their application in every day clinical practice (65).

CLINICAL UTILITY OF BONE BIOCHEMICAL MARKERS

Monitoring anti-osteoporosis therapeutic efficacy and compliance

Perhaps the greatest part of our knowledge on the clinical utility of bone markers is based on the results achieved in various pharmaceutical studies on novel anti-osteoporotic drugs (68-78). The nature of their change in response to treatment is well characterized, and can be utilized to forecast increases in bone mineral density and therapeutic efficacy in reducing fracture risk.

Anti-resorptive drugs sabotage bone resorption by driving osteoclasts into apoptosis and hence resulting in rapid decrease in bone resorption marker levels. The dose and mechanism of action dictate the degree of inhibition of bone resorption, as such the resorption marker levels. Since bone resorption and formation are coupled processes, inhibition of bone resorption results in decrease of bone formation, as such decrease in bone formation marker levels.

It is now evident that following patients every 3-6 months with their bone marker levels can monitor drug adherence and efficacy. This is an advantage over bone mineral density examinations where a follow-up of within 1-year period is not productive, since changes in BMD take longer to happen than bone markers, follow-up DEXA scans are limited by their estimation of least significant change (LSC) and BMD changes explain only a part of fracture reduction (66,68). Table 2 summarizes the changes in bone marker levels during different anti-osteoporosis therapeutic regimes. Treatment with anti-resorptive drugs, e.g., estrogen, selective estrogen receptor modulators, like raloxifene, bisphosphonates, like alendronate and risedronate, and denosumab are associated with decrease in markers of bone resorption and formation. Human recombinant PTH treatment is associated with increase in bone formation markers followed by increase in bone resorption marker levels (74).

Both baseline bone marker levels and changes following initiation of therapy predict BMD changes. Early decrease in bone marker levels with bisphosphonates and denosumab are known to correlate with 2-3-year increases in BMD (70, 71).

Fracture risk reduction at the spine and hip has been reported where early marker level reduction (P1NP, BSAP, CTx) was demonstrated upon commencing alendronate therapy (70). Raloxifene induced changes in osteocalcin predicted spine fracture risk reduction better that changes in BMD (79, 80). Similar association was observed also between P1NP and zolendronate (81).

Predicting bone loss and fracture risk

It has been recognized, based on population-based studies, that elevated marker levels predict accelerated bone loss and increased non-traumatic fracture risk independent of underlying co-morbidities, age or sex (82,83). Nonetheless, the implementation of these population-based observations has been difficult on the individual patient level. Prospective randomized clinical trials designed to assess the efficacy and cost-effectiveness of screening programs are

Table 2 Changes in bone biomarker levels during different anti-osteoporosis therapeutic regimes

Bone marker	Type	Therapy	Target levels	Follow-up period
ß Crosslaps	Resorption marker	Anti-resorptive	min. 35% ↓	Baseline and every 6 months
Total P1NP	Formation marker	Anti-resorptive	min. 40% ↓	Baseline and every 6 months
		Anabolic	min. 40% ↑	Baseline and every 6 months
Osteocalcin	Turnover marker	Anti-resorptive	min. 20% ↓	Baseline and every 6 months

missing, and currently use of bone marker measurements are not recommended to identify patients at increased risk of bone loss as a public health measure (84).

Use in Nephrology

End-stage renal failure is usually associated with renal osteodystrophy. The hallmarks of renal osteodystrophy include low serum calcium levels and elevated PTH (secondary hyperparathyroidism). The high bone turnover characteristic of the condition results in high bone turnover marker levels. TRAP and BSAP are the only biomarkers not cleared by the kidney and as such reflect the state of bone turnover. Monomeric P1NP, osteocalcin and CTx typically are elevated, and these elevations do not reflect the true nature of bone turnover. PTH levels are known to show good association with bone turnover (85). Practically speaking serum BSAP ad PTH are the only reliable marker in patients suffering from kidney disease.

Use in Oncology

Solid tumors like prostate, lung and breast cancer typically metastasize to the bone. Furthermore, primary involvement of bone is characteristic of multiple myeloma. Depending on the tumor, the bone involvement may be osteolytic or osteoblastic. This potentially suggests that resorption marker elevations dominate during osteolytic presentation and formation markers are elevated when osteoblastic bone lesions are manifested (86). Furthermore, bone biomarkers function as tumor markers when secreted directly by primary bone tumor. Osteoid osteoma secretes osteocalcin (87). BSAP can be secreted by osteosarcoma (88).

Use in Rheumatology

The inflammatory pathogenesis of most rheumatological disorders promotes bone resorption and suppresses bone formation. This uncoupled constellation in marker levels has been reported in rheumatoid arthritis, polymyalgia rheumatic, psoriatic arthritis, ankylosing spondylitis, and reactive arthritis (89,90). Furthermore, the resorption marker levels usually show good correlation with disease activity indices of different rheumatological conditions (91-93).

Use in Paget's disease of the bone

Paget's disease of the bone is bone metabolic disorder characterized by extremely high bone turnover causing expansion and deformation of affected bones. Patients usually present with marked increase in all bone turnover marker levels (94). P1NP concentrations have been demonstrated to correlate with disease activity and to anti-resorptive therapeutic response, as such bone turnover markers present traits of both diagnostic and disease monitoring in Paget's disease (95).

CONCLUSION

Biochemical markers of bone turnover reflect bone homeostasis, i.e., the activity of osteoblasts and osteoclasts in both physiological and pathophysiological conditions. Although quite sensitive to a multitude of exogenous and endogenous pre-analytical factors, bone markers are best used in monitoring anti-osteoporosis therapy efficacy and compliance. Combination of BMD measurement by DEXA with biochemical markers of bone turnover levels, at least one bone resorption and one bone formation marker, may potentially improve early detection of individuals at increased risk for bone loss and eventually non-traumatic bone fracture. Furthermore, they have widespread clinical utility in osteoporosis, renal osteodystrophy, certain oncological conditions and rheumatic diseases.

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