Biochemical markers in the follow-up of the osteoporotic patients

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Summary

Osteoporosis, a disease characterised by low bone mass and micro-architectural deterioration of bone tissue, is viewed as an emerging medical condition. Bone mineral density (BMD) is considered the gold standard of bone status assessment, however it does not offer the timely response desirable for monitoring. Biochemical markers of bone turnover (BTMs) are claimed to be suitable for that purpose. There is not generalized agreement on which marker could be used in routine. The present paper reviews pros and cons of currently used BTMs and relative analytical methods. Several analytical issues, such as biological variability, molecules stability, lack of reference materials jeopardize the field and, consequently, recommendations are difficult to be drawn. Reference range can't be used to support clinical judgement and, in this view, Least Significant Change (LSC) is regarded as a way to improve the interpretation of analytical results.
Bone alkaline phosphatase (bALP) is still a marker of interest and its use is widespread in clinical laboratories; Tartrate Resistant Acid Phosphatase band 5b (TRAP 5b) appears to be a promising marker. N-terminal propeptides of type I collagen (s-PINP) and beta-collagen 1 C-terminal cross linked telopeptides (s-CTX), given low biological variability and assay availability for automatised instruments, should be the marker of choice in future clinical trials, to overcome the paucity of uniform data and should be used in clinical routine, to monitor osteoporosis treatment. Finally, the lack of standardisation of currently available diagnostic methods, could be overcome by harmonisation.

KEY WORDS: Bone Turnover Markers; osteoporosis.

Introduction

Osteoporosis is defined as a disease characterised by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced bone fragility and consequent increase in fracture risk (1). Osteoporosis is identified as one of the emerging medical conditions requiring attention during the 21st century, due to its clinical consequences, such as hip fractures and related human and economical costs.
Bone mineral density (BMD) is the gold standard of bone status assessment in osteoporosis, leading to internationally applied diagnostic criteria.
However, BMD does not offer the timely response desirable for monitoring therapeutic response (2). Biochemical markers of bone metabolism offer the potential for screening bone turnover conditions, as well as for monitoring early response to therapy, providing a rationale for their use to monitor treatment in a clinical setting (3). The importance of markers in osteoporosis was recently reviewed by Bouxsein and Delmas (4).
An ideal marker must have specific characteristics: biological plausibility (i.e., relation between biomarkers and pathogenetic mechanisms leading to increased skeletal fragility); association between biomarker and fracture in the target population; consistent change in response to therapy. This latter, possibly, in a predictable and dose-dependent fashion that underlies known mechanism of action of therapeutic intervention. Eventually changes in biomarkers with treatment must account for a substantial proportion of the antifracture efficacy.
Bone turnover markers (BTMs) are biochemical products, measured usually in blood or urine, that reflect the metabolic activity of bone. It needs to be stressed that markers themselves have no function in controlling skeletal metabolism.
They are traditionally categorised as markers of bone formation or bone resorption (3). Resorption markers are either osteoclastic enzymes or products of collagen degradation, released into the circulation. Formation markers are either osteoblastic enzymes or breakdown products of collagen synthesis or matrix proteins.
The present review focuses on the most common bone turnover markers used in clinical practice and, based on the best available evidences, suggests how to use them in the management of osteoporotic patients.

Bone Formation Markers

Bone Alkaline phosphatase
Bone Alkaline phosphatase (bALP), introduced into clinical use in 1929, was the first biochemical marker of bone turnover and is still the one most widely used. Bone alkaline phosphatase has a molecular weight of approximately 140000 Dal and is found in the membrane of osteoblasts. It is released into the circulation during bone formation.
This marker is very stable in blood samples and it is not affected by haemolysis. Currently used assays can detect the bone isoform of alkaline phosphatase (5). Since alkaline phosphatase is produced by different cell type, resulting in different carbohydrate content, relatively specific immunoassays for bALP from bone were developed, although cross-reactivity of up to 20% between the bone and liver enzymes (6) is still present in all assays.
The long-term intra-individual variability of bone alkaline phosphatase is up to 10%, and such biological variability represents the major component of total variability, since the improvement of analytical methods.

Osteocalcin
Osteocalcin is a large peptide synthesized by osteoblasts, odontoblasts, and some chondrocytes. It binds to hydroxyapatite and it is deposited in the bone matrix. As osteocalcin fragments are released from the bone matrix during resorption, assays for circulating osteocalcin and its fragments reflect both bone formation and resorption (7). Only a small fraction of osteocalcin is released into the circulation following a circadian rhythm peaking at 4 a.m. It is cleared by the kidney and its levels are affected by renal impairment. Osteocalcin has a half-life of less than one hour and it is quickly degraded; the intact molecule and fragments coexist in circulation (8). The presence in variable amount of different fragments introduces several analytical problems, not to mention the assay's lack of standardisation (9) . Furthermore, the serum degradation, even in the absence of haemolysis, causes an important preanalytical problem, making comparison between different assays even more difficult.
Given all the above mentioned facts (10) osteocalcin cannot be considered optimal in routine clinical practice.

Procollagen I extension peptides
Type I collagen is synthesized by osteoblasts as the precursor molecule procollagen, with extension peptides in the carboxy (C) and amino (N) ends. These extensions are cleaved by proteases during collagen extra cellular metabolism, producing N-terminal (PINP) and C-terminal (PICP) propeptides of type I collagen. They are found in blood as a trimeric form, rapidly converted in a monomeric form and represent a marker of type I collagen synthesis (11). Different assays can measure both monomeric and trimeric forms. The clearances of the two forms are most probably different, according to recent literature. Intact PINP is mainly metabolized by the endothelial cells of the liver whereas clearance of monomeric PINP depends on kidney function. PINP measurement has the practical advantage of a low diurnal variability, and its circulating levels are not significantly influenced by food intake (patient does not need to be fasting) (12, 13).

Bone Resorption Markers

Hydroxyproline
Hydroxyproline is an amino acid common to and characteristic of all forms of collagen. Urinary hydroxyproline excretion is the oldest test of bone resorption. However, its lack of specificity is well recognized: excreted hydroxyproline may originate from skin collagen (which can turn over rapidly in certain disorders), from newly synthesized collagen not incorporated into tissue, and even from dietary collagen and gelatin. Hydroxyproline measurement is no longer recommended (14).

Pyridinium cross-links
Pyridinolines are cross-linking amino acids that strengthen collagen fibrils in the extracellular matrix. They are found in the main fibril-forming collagens (types I, II, and III) of many tissues. Pyridinoline is the major chemical form, but deoxypyridinoline is also abundant in bone collagen and it is considered to be a relatively selective bone marker. Assays are currently available for serum and urine samples. These markers follow a circadian rhythm and are higher early in the morning and scarcely influenced by diet. To date immunoassays are widely used as alternative to high performance liquid chromatography (HPLC) (15, 16).

Telopeptides of type I collagen
These peptides are the non-helical region of type 1 collagen where the crosslinks attach. The measured molecules are either a trimeric carboxyterminal telopeptide (ICTP), which is measured in serum by radioimmunoassay (10) or the aminoterminal region (NTX) or the carboxy-terminal region (CTX). They are produced by osteoclasts during bone resorption. NTX and CTX can be measured in either serum or urine. The serum levels are influenced by circadian rhythm and food intake, therefore samples must be collected at a given time of the day (preferably in the morning) and fasting (17). The 24-hour urine collection has the advantage of overcoming circadian changes and is less sensitive to dietary interferences, although proper urine collection may be troublesome for the patients.
A further group of collagen decomposition products has gained attention over the last years: fragments with telopeptides including specific epitopes, such as: beta-collagen 1 C-terminal cross linked telopeptides (beta-CTx) and beta-Crosslaps.
Beta-CTx and beta-Crosslaps assays recognize fragments of collagen 1 that have the beta isomerized 8AA-octapeptide (EKAHD-beta-GGR) which builds an epitope located on C-terminal telopeptides. Often the terms beta-CTx and beta-Crosslaps are used synonymously. However, there is a small, test dependent, difference: Crosslaps includes fragments that containing at least one 8AA peptide 6,7; beta-CTx includes fragments containing at least two 8AA-peptides (18).
Assays directed towards the 8AA peptided are known to be more bone specific; serum assays, in particular, detects two 8AA resulting more specific compared to urine assay that recognises only one 8AA (19).
Automated CTX serum assay has become increasingly available and it's replacing urinary NTX because of its simplicity and robustness. Reference intervals should be age and sex-specific.

Other markers of bone resorption
Two enzymes found in osteoclasts have received attention as markers of osteoclast activity.
Osteoclasts produce an acid phosphatase isoenzyme which is not inhibited by tartrate, called type 5 Tartrate Resistant Acid Phosphatase band 5b (TRAP 5b). This enzyme is present in the osteoclast's ruffled border membrane and in the resorptive space. Increased TRAP 5b levels have been described in high bone turnover states, like Paget's disease and bone metastasis. Recently, due to assay evolution, TRAP 5b is becoming one of the BTM used for prediction of high bone turnover significantly related to BMD loss (20). Few studies on type 5b TRAP in osteoporosis affected patients have been reported.
Serum cathepsin K is of interest because it is the primary proteolytic enzyme used by osteoclasts to degrade bone type I collagen during resorption. Although several studies suggest that it may be a valuable marker of bone resorption (21), more trials are required to evaluate its performance.

Recent advances in research
Due to the paramount interest in this field, researches have been encouraged to identify new potential BTMs. Among these, the following must be mentioned: receptor activator of nuclear-factor kappa-B ligand (RANKL) and osteoprotegerin (OPG), two cytokine of the tumour necrosis factor (TNF) family, are osteoblasts products. Receptor activator of nuclear-factor kappa-B (RANK) is localized on the surface of osteoclasts and pre-osteoclasts.
Bone resorption is influenced by osteoblasts through the interaction between RANK, RANKL and by the OPG that inhibit RANK–RANKL interaction (22-25). OPG and RANKL play a critical role in the regulation of bone turnover acting on osteoclast activity. The circulating levels of OPG and RANKL are inversely related to BMD and contribute to the development of osteoporosis in postmenopausal women (26). They may possibly be used as markers of bone metabolism, although the broad role of RANK ligand signaling in the immune system may limit its specificity.

Sources of variability

To avoid being mislead, clinicians who use biochemical markers

of bone turnover should be familiar with factors that influence BTMs and, in turn, assay results (27).
The most important biologic factors are diurnal and day-to-day variability in bone forming and bone-resorbing activities. Levels of bone turnover markers are highest in the early morning and lowest in the afternoon and evening (28). Levels of urinary markers can, accordingly, vary up to 30% during the day. It is worth to mention that the expression as a ratio to creatinine, intended to limit such variability, may introduce, in turn, another bias (10).
Fasting blood samples should be obtained in early morning. An increase in dietary calcium intake can lower the levels of bone resorption markers, particularly in people whose calcium intake was previously low (29). Presumably, this effect is mediated by inhibition of parathyroid hormone secretion. In addition to all the mentioned issues, preanalitycal conditions are known to be critical: collection, transport, centrifugation and storage should be performed within 4 hours, in refrigerated conditions for most of them.
Several factors such as age and sex (children and post-menopausal women), but also physical activity, can increase bone turnover (30); reference ranges, adjusted for age and sex, are recommended but unfortunately, this information is not always available (31, 32).
The lack of assay standardisation is still a matter of concern, making difficult the comparison of results obtained by different methods and/or in different laboratories. This is the reason why the Consensus of the Belgian Bone Club suggests that patient's monitoring should be always done in the same laboratory (10).
The use of BTM has further limitations: long-term corticotherapy, limited mobility, bone metastases, acromegaly and thyrotoxicosis may change bone turnover (33). In particular, the use of corticosteroids exceeding 3 months, inhibit bone formation with a fall in osteocalcin, PINP and ALP and increases bone resorption (34, 35). Time plays an important role, when bone methabolism is concerned: bone formation and resorption markers increase as early as a minimum of 4 months after the fracture, reflecting bone healing (36).

The potential use of BTM as a tool to assess fracture risk and to monitor treatment

Fractures risk prediction and BTMs: the currently available evidences

Several prospective cohort studies showed that markers of bone formation or resorption are significantly associated with fracture risk (37-39). Moreover, when women with a low BMD have increased levels of BTMs, fracture risk is further increased. Although men are less extensively investigated, several studies suggest that BTM plays a role in fracture risk prediction. A key point is the definition of the best biomarkers to be used in clinical practice; in this view, a systematic review of all the available data would be of great help. Unfortunately, given the heterogeneity of published data, due to different population, type and number of markers used for monitoring, length of follow up, choice of treatment, just to mention only few of the methodological issues, such filtered information is not yet available and clinicians need to relay on indications derived from primary studies.
Nevertheless, the following data (3) are of some interest: u-CTX appears to be an independent factor (i.e. not related to BMD value) to define hip fracture risk in women; a decrease in carboxilated s-OC/total s-Oc ratio is associated with increased risk of subsequent fracture, in men; the serum increase of s-ICPT is associated with an increased risk of osteoporotic fractures independent of BDM in the male Australian population.
We can affirm that although BTMs and particularly those of bone resorption, may have some utility in predict fracture outcome, a clear conclusion cannot be drawn yet (3).

Monitoring of osteoporosis treatment and BTMs: the currently available evidences

Clinicians are in great need of a tool to monitor osteoporosis treatment in order to choice the best therapy, the best dose and the optimal dose frequency. As previously mentioned, BTMs have been considered the ideal choice, compared to BMD, given the rapid changes following therapy.
Markers of bone resorption decrease within days or weeks of starting treatment with antiresorptive agents.
Although there is a general agreement on the rational for BTMs use, is not easy to interpret clinical research aimed to define its routine use. Many variables need to be taken into account: mechanism of action of the drug (antiresorptive versus anabolic), drug doses, route of drug administration, specific response of the single marker. As a general rule, a baseline assessment is required, followed by repeated measurements at some times during treatment.
The extent of the observed change, in turn, is influenced by drug efficacy (level of change) and by imprecision of the measurement and, finally, by intra-individual variability. The concept of Least Significant Change (LSC) was introduced (40) in order to be confident that a consistent change in markers value has occurred. The key point is, however, to observe a change in the primary outcome: the number of fractures. Several trials indicate that the larger the decrease of in BTMs, following anti- resorptive treatment, the larger the reduction in fracture risk (Table 1). The FIT trial (37), the HORIZON (38) trial, the MORE (39) trial report the decrease in BTMs following treatment, expressed as Odds ratio or Hazard ratio. Interestingly, although all of these trials focused on different drugs (alendronate, zoledronic acid, raloxifene respectively) the magnitude of the effect for each marker and for each marker type (resorption or formation) was similar (ranging from -59.2% to -40.8% change in BTM).

Interpretation of BTM

The treatment of osteoporosis induces large and rapid changes in BTMs. Several studies have described a significant relationship between the reduction in BTMs following anti-resorptive therapy and the reduction in vertebral and non-vertebral fracture risk (37-39, 41, 42), supporting the use of BTMs for monitoring osteoporosis treatment (43). A baseline assessment with repeated measurements at defined points during therapy is mandatory. In order to effectively use markers, it is important to appreciate the LSC: only a decrease higher than the LSC can be interpreted as a potential biological effect (44).
Recent guidelines have suggested that a decrease of at least 30% for serum markers and 50%-60% of urinary markers need to be documented in order to suggest that a biological event has occurred.
The ability to detect changes between the two values with confidence is also related to the imprecision of the measurement, as well as biological (intra-individual) variability, which may be influenced by factors such as time of day, fasting, compliance to instructions.
Many studies have shown that the intra-individual variability is around 10% for serum markers and 30% for urine markers, and the signal-to-noise ratio is better for serum markers (10), so serum samples are preferred to urine samples when measuring BTMs.
This indication, as well as the need to define age and sex related reference ranges, has important consequences for follow up.
The LSC for each BTM, considering the within-subject and between-subject variation, need to be accurately defined.
Serum CTX and s-PINP show responsiveness to treatment and low within-subject variability. Thus, their measurement usually enables the identification of the majority of responders to treatment using the LSC approach (45, 46).
Laboratories must guarantee that the analytical variability is well documented and under control to minimize the contribute to marker LSC.

Table 1 - The performance of BTMs in monitoring treatment.

Treatment	Trial	Author	Sample size[a]	Sample size (fractures)	BTM	Measurement of BTM (months)	% change in BTM	Follow-up for fracture (years)	Fracture endpoint	Outcome (95%CI)
Alendronate	FIT	Bauer 2004 (37)	6,087	(336)	s-PINP	12	–50.9 SD 30.7	Mean 3.6	Vertebral	OR 0.77 (0.66-0.90)
				(515)	s-PINP	12	–50.9 SD 30.7	Mean 3.6	Non-vertebral	RH 0.90 (0.80-1.03)
				(46)	s-PINP	12	–50.9 SD 31.1	Mean 3.6	Hip	RH 0.78 (0.51-1.19)
			6,142	(336)	s-CTX	12	–59.2 SD 31.1	Mean 3.6	Vertebral	OR 0.83 (0.73-0.95)
				(515)	s-CTX	12	–59.2 SD 31.1	Mean 3.6	Non-vertebral	RH 0.94 (0.84-1.06)
				(46)	s-CTX	12	–59.2 SD 31.1	Mean 3.6	Hip	RH 0.89 (0.61-1.31)
			1114		s-CTXc	12	–53.3 SD 35.5	Mean 3.6	Vertebral	OR 0.77 (0.58-1.03)
					s-CTXc	12	–53.3 SD 35.5	Mean 3.6	Non-vertebral	RH 1.02 (0.75-1.37)
Zoledronic acid	HORIZON	Delmas 2009 (38)	1,270	(130)	s-PINP	36	–56	3	Any clinical	RR 0.62 (0.43-0.88)
				(114)	s-PINP	36	–56	3	Non-vertebral	RR 0.67 (0.46-0.98)
				(20)	s-PINP	36	–56	3	Hip	RR 0.43 (0.17-1.13)
				(21)	s-PINP	36	–56	3	Vertebral	OR 0.32 (0.11-0.86)
				(40)	s-PINP	36	–56	3	Vertebral	OR 0.17 (0.07-0.41)
Raloxifene	MORE	Reginster 2004 (39)	967		s-PINP	12	–40.8	3	Vertebral	Slope 0.0085 (0.0021-0.0150)
					s-PINP	12	–40.8	3	Vertebral	TEE 27.5 (3-51)

Vasikaran et al. (3), modified, for more details see the original tables.

OR = odds ratio per 1 SD decrease in BTM; RH = relative hazard per 1 SD decrease; TEE = treatment effect explained (percent); Slope = slope from log regression analyses of vertebral fracture risk and percentage change in BTM.

Conclusion

The potential use of BTMs to predict the response to treatments for osteoporosis in the individual patient is of great interest. Treatment-induced changes in specific markers account for a substantial proportion of fracture risk reduction (3).
Urine markers were valuable tools before the advent of serum markers; today several bone markers can be determined in blood, reducing significantly the variance due to 24h urine collection.
For these reasons the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) have launched in 2009 the IOF–IFCC Bone Marker Standards Working Group, that has recommended to choose s-CTX and s-PINP as the analytes of choice for bone resorption and formation, respectively. Such molecules should be used in future clinical trials and observational studies in osteoporosis, in order to enhance laboratory consistency, enlarge the international experience of the clinical application of BTMs to osteoporosis and facilitate their inclusion in routine clinical practice (43). The standards used in these assays are well characterised; furthermore, automated immunoassay are already available for these tests.
The biological and analytical variability of s-CTX an s-PINP have been well documented as well as the requirements of sample handling and stability.
Very recently the Vasikaran et al. position paper was criticised by Seibel (47). The author expresses doubts on the opportunity to dismiss bALP and ICTP; in his view the consensus paper did not bring strong enough arguments to support such a statement. In fact the preanalytical variability of sTRAP5b is minimal and considerably lower than that of other markers, which results in a very promising signal to noise ratio (48). As far as ICTP is concerned, some data indicate that it is predictive of fractures in men, whereas sCTX-I is clearly not (49). An interesting remark made by Seibel is the potential conflict of interest arising from the presence of just one company manufacturing the two selected markers (sCTX and s PINP). Although it must be acknowledged the issue soundness, we can suppose that the growing request of biological markers will stimulate other companies to update their offer, as already seen in different laboratory medicine fields. Seibel finally recalls an issue of paramount importance: which is the LSC in marker level to be consider a “true response”? Vasikaran (3) arose the same question in his work, but unfortunately to date the answer is lacking, given the poor number of clinical trials addressing the specific issue and it could remain a unsolvable problem, due to high degree of interindividual variability as previously mentioned.

Future development

Over and above the identification of the reference BTMs, an important further step is to standardise the measurement of each marker to obtain comparable values, irrespective of the laboratory in which the measurement is made, as well of the method utilised (50).
Moreover, the use of internationally accepted decision limits and target values requires that measurements are universally comparable.
Standardisation and the establishment of a reference system (51) for the BTMs is the route to achieve this.
Standardisation requires a reference method, high quality reference materials and validation of proper method calibration through appropriate traceability to reference methods.
The steps towards obtaining international standardisation of assays can be slow and laborious. It is possible that a strategy of partial harmonisation of assays could be adopted as a short-term *ad interim* solution.

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