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Bone turnover markers in clinical practice and their potential use in HIV-related bone disease

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Key words: osteoporosis; HIV; bone turnover markers

ABSTRACT

Bone metabolism is characterized by two opposing activities coupled in time and space in the so-called ‘bone remodelling units’. Through a continuous remodelling cycle old bone is resorbed by osteoclasts while osteoblasts deposit new bone. Bone loss is due to an imbalance between bone resorption and formation. Biochemical markers provide a dynamic view of the remodelling process, which can improve fracture risk prediction. Furthermore, they can be used to monitor the short-term effects of therapy, treatment efficacy and patient compliance. Markers of bone remodelling can be dosed in plasma and/or urine, as indicators of osteoblast function or osteoclast function. The significance of any bone turnover marker (BTM) depends on two fundamental characteristics: specificity and variability. The biological variability may determine some limitations in the interpretation of the data. Even though some uncertainties on how to interpret a given result are not resolved as yet, it is impossible to ignore the utility of BTM. As they are easy to obtain and inexpensive compared with other not always mandatory examinations, it seems justified to use BTM in our clinical practice. HIV-related bone disease is characterized by a high turnover state, with an exaggerated osteoclastic bone resorption and delayed osteoblastic bone formation. Few data exist on the relevance of BTM in HIV-infected patient management. Following the considerations drawn for the general population we suggest that, at least in osteoporotic HIV-patients, BTM be used both to increase the ability of fracture prediction and to monitor the response to anti-resorptive therapy.

Introduction

Bone tissue is subject to remodelling throughout the lifetime of an individual. Through a continuous remodelling cycle, that takes place inside the so-called ‘bone remodelling units’, old bone is resorbed by osteoclasts with the formation of cavities that are subsequently filled by osteoblasts. The result of this combined action of osteoclasts and osteoblasts is the replacement of old bone with new bone. Bone loss observed in old age and in women after menopause is caused by a imbalance between bone resorption and deposition. The lack of oestrogens increases osteoclast activity and therefore the number of ‘bone remodelling units’ with an imbalance between bone resorption and formation. This therefore leads to a progressive, sometimes rapid, loss of bone mass [1-3]. It is however accepted that the development of osteoporosis is conditioned not only by the rate of bone loss, but also by having reached a low peak in bone mass [4]. Both these parameters are determined by numerous factors deriving from the interaction between the individual’s genetic characteristics and environmental conditions.

Currently available methods for studying bone metabolism are a) the measurement of bone density; b) histomorphometry of biopsies; c) biochemical markers of bone turnover.

Bone mineral density is a static parameter useful for evaluating the risk of fracture but it does not provide any information on bone turnover, or the pathogenesis and short-term efficacy of drugs. Histomorphometry on biopic samples provides information on turnover and pathogenesis, but is a site-specific and invasive technique that does not easily lend itself to routine use. This effectively leaves only the use of biochemical markers of bone metabolism as the method of choice in clinical practice. Biochemical markers provide a dynamic view of the remodelling process, which covers rate of turnover and pathogenesis, and should improve fracture risk prediction [5]. They can also direct the clinician to alternative diagnoses, since the values deviate excessively from reference levels [6]. Furthermore, they can be used to monitor the short-term effects of therapy, and indicate if an excessive slowing of the remodelling process is occurring.

When searching for markers of bone remodelling, biochemists have focused mainly on skeletal molecules that can be dosed in plasma and/or urine (Table 1):
a) osteoclast and osteoblast related enzymes  
b) collagen fragments  
c) non-collagen components of the intercellular matrix.

From a clinical standpoint, one should then examine what the characteristics of the ideal marker should be:
- bone specific  
- specific for resorption or formation  
- a usefully long half-life  
- resistant to degradation  
- completely expelled in an unmodified form, for measurement in urine  
- sufficiently sensitive and reproducible as measured by a low cost method

It would also be necessary to define the optimal conditions for harvesting and preserving samples to minimize the analytical variability of the method, and to verify the biological variability within and between individuals in both the short and long-term.

This paper reviews currently available bone remodelling markers, benchmarking their characteristics to those of the ideal marker.

**Bone Formation Markers**

**Alkaline Phosphatase (AP)**
This enzyme catalyses the hydrolysis of phosphoric acid esters and it is important not only in bone calcification processes, but also in carbohydrate and lipid metabolism. The four most clinically significant isozymes originate in the liver, bone tissue, intestine and placenta respectively. Excluding pregnancy, and given that the sample is collected after fasting, the measurable activity in plasma is almost totally of hepatic or skeletal derivation. This parameter can be very useful in diagnosing skeletal diseases such as osteomalacia, Paget’s disease of bone, primary hyperparathyroidism and bone metastases.

Instead, total AP levels do not correlate with the common histomorphometric indices for osteof ormation [7], and total AP is a poorly sensitive and specific index of pathology in osteoporosis. To increase the sensitivity and specificity of the test, techniques have been developed to identify the bone AP isozyme (BAP). Both bone and liver isozymes are coded by the same gene and they differ only by post-translational modification, leading to various degrees of glycosylation [8]. Monoclonal antibodies that recognise the bone AP with a cross-reactivity with the liver AP of less than 9% have recently been obtained [7, 9]. Despite the relatively long half-life of bone AP (1-2 days), this assay is carried shortly after collecting the sample, which must be stored at -20°C as enzymatic activity increases at higher temperatures.

**Osteocalcin**
Also known as bone γ-carboxyglutamic acid (Gla) protein, osteocalcin (OC) is the principal non-collagen protein of bone, whose synthesis by osteoblasts and odontoblasts is regulated at the gene level by 1-25(OH)2D3. Once synthesised, it binds to the hydroxyapatite in bone matrix, due to the high affinity for calcium of the Gla residues, while only a minor fraction is released into the circulation where it can be measured using immunoaffinity methods [10,11].

This non-binding portion is quite variable, ranging from about 10% in children up to about 30% in adults.

Measuring this protein presents some serious limitations deriving from its short half-life, in the order of a few minutes [12], its rapid renal clearance, and the fact that it is quite fragile, being subject to fragmentation. For this reason, it should be measured immediately after sample collection, as in vitro serum degradation reduces immunoreactivity by over 90%. Currently available polyclonal antibodies, directed against bovine osteocalcin but cross-reacting with the human protein, are capable of recognising fragments as well as the intact molecule. Despite its high specificity, the clinical use of this molecule is rather disappointing, due to the extreme variability of its measurements.

**Type I Collagen Carboxyterminal Peptide**
Type I collagen makes up over 90% of bone organic matrix. Although it is present in almost all the connective tissues, the bulk of this molecule occurs in

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S= serum  
U= urinary
Bone and skin. The bone collagen is synthesized as a precursor (procollagen) with propeptides at both the N- and C-termini. These peptides, which assist procollagen assembly within osteoblasts, are proteolytically removed on secretion and thus they are not incorporated into the existing matrix. Given the fixed ratio existing between collagen fibrils and released propeptides, numerous studies have considered these peptides as possible markers for bone formation. The C-terminal propeptide has mostly been measured, whereas the use of the N-terminal propeptides is rarer [13]. The C-terminal propeptide has a half-life of only 6 to 8 minutes, probably due to a rapid, receptor-mediated uptake by liver endothelial cells [14]. Currently available assays show a considerable variability in measurements of this molecule, likely due both to the intrinsic variability in type I collagen synthesis and to the rapid metabolic clearance of the propeptide.

**Bone Resorption Markers**

**Tartate-Resistant Acid Phosphatase**
This isoenzyme of acid phosphatase is released by osteoclasts during the bone resorption phase. It is also produced by other cells, such as macrophages [15]. No data are yet available on its use as a clinical marker.

**Hydroxyproline**
Hydroxyproline (Hyp) is a post-translationally hydroxylated amino acid, which is almost exclusively present in type I collagen, except for a certain amount present both in the C1q fragment of complement and in elastin. The concentration of Hyp in urine can thus be used as an index of bone turnover as it increases during resorption due to collagen degradation. The most commonly used method for its measurement is based on a colorimetric determination, although data obtained using ion-exchange or gas-liquid chromatographies are more reproducible [16]. Fundamental aspects in data evaluation are dietary interference and the biological variability of this marker. About 85-90% of Hyp released by tissue is metabolized so that only 10-15% is excreted in urine, and of this only about 10-15% derives from newly synthesized collagen or from non-bone collagen molecules.

**Pyridinoline**
Collagen fibrils consist of molecular peptide subunits each of which is formed by three supercoiled peptide chains. One of the fundamental steps in the assembly of fibril is the formation of covalent cross-links, which can occur both within a given collagen fibril and between adjacent fibrils that are essential for the stability of the final protein structure. These cross-links form between three lysine-derived residues, two of which are Hyl residues at the N- or C-termini of the peptide chains (the telopeptides), the third being a Hyl or Lys residue in the central helical portion of an adjacent fibril molecule. They give rise to the characteristic ring structures known as pyridinoline nuclei. These are present in two forms: hydroxyypyridinoline (pyridinoline, Pyd) and deoxyypyridinoline (lysyl-pyridinoline, Dpd). Pyridinolines, which are minimally metabolized and subject to negligible hepatic excretion, can thus be considered as final products of collagen catabolism. They are excreted in urine in the free form (40%), or linked to collagen peptide fragments of varying molecular weight (60%). While Pyd is distributed among all connective tissues, Dpd is found predominantly in bone [17, 18].

Pyridinoline levels are very high during growth, reach a plateau in adulthood and then increase by about 50-100% in women after menopause. They decrease to pre-menopausal levels with estrogen therapy [19] and they correlate with bone turnover in osteoporotic patients [20].

Pyridinoline cross-links can be measured in urine via high resolution liquid chromatography. However, to increase the reproducibility of measurements, and to take into account the relatively high pre-analytical variability, methodologies using polyclonal antibodies have been developed that can recognize both Pyd and Dpd, as well as an immunoassay that exclusively recognizes Dpd [20].

**Collagen Telopeptides**
As mentioned above, the N- and C-terminal extremities of the peptides making up a collagen fibril are involved in the formation of pyridinoline cross-links. Collagen catabolism results in a series of peptide fragments that are linked to pyridinoline or deoxypyridinoline moieties, and if these derive from either of the termini, they are respectively denoted as the amino-terminal (NTx) or carboxy-terminal telopeptides. An eight fragment of the latter telopeptide, characterized by the presence of a β-aspartate residue, is denoted CTx. This residue differentiates CTx from an analogous fragment derived from skin collagen, in which an α-aspartate residue is present.

More recently, monoclonal antibodies have been developed that can recognize cross-links in both NTx and CTx [21]. Due to the presence of a β-aspartate residue only in bone telopeptides the CTx determination is highly specific.

**Choosing a Marker**

The clinical significance of any marker for bone remodelling depends on two fundamental characteristics: specificity and variability. The specificity indicates the capacity of the marker to reflect variations that derive exclusively from biological activity in the skeleton. Dpd, for example, is not present in skin and the amount deriving from other extra-ostea tissue is negligible, so that it is undoubtedly a specific marker [22]. Bone AP, being an expression of only osteoblast activity is a highly specific marker for bone neo-position. The telopeptides deriving from enzymatic cleavage of the N- and C-terminal extremities of type I collagen, that is also present in the skin, are instead less specific.

The problem of marker variability is more complex, given that quite sizeable variations in measurement may not have a clinical significance, but rather be completely justified simply by pre-analytical and/or analytical variability. These could derive not only from different conditions in the sample collection, or from different methodologies used for obtaining the measurement, but also, and often mainly, from an intrinsic biological variability that is tied to different factors such as age, sex (non controllable factors) and diet, fasting and circadian variations (controllable factors). Osteocalcin, for example, is characterized by...
a high intrinsic pre-analytical variability of biological nature, but it is also subject to high analytical variability, as it exists as various fragments, each of which is recognized differently by the different commercially available diagnostic kits.

The ‘biological variability’ underlying the pre-clinical variability can be distinguished into two components: the ‘intraindividual variability’, due to the normal fluctuation of a biological parameter when measured at different times in the same individual, and the ‘interindividual variability’, due to different expression of a biological parameter in different individuals in the same conditions. Taking these considerations into account, the ‘critical difference’ is the percentage difference required to consider two consecutive determinations of a marker in the same individual significantly different. From a clinical perspective it is clear that the biological variability needs to be known. The clinician is facilitated by choosing markers with the least possible biological variability. From this standpoint serum markers show a lower individual variability.

Factors that are not easily modified are further confounding sources. In postmenopausal and elderly women, the major uncontrollable factors are diseases and associated bed rest and immobility, nutritional status, and recent fractures. Several diseases can influence bone turnover markers (BMT). Bone formation and resorption are mildly decreased in diseases characterized by low bone turnover such as hypothyroidism and hypopituitarism, whereas they are increased in Paget’s disease and primary hyperparathyroidism, that are characterized by an accelerated bone turnover.

In addition, many drugs can interfere with bone metabolism, affecting BMT. The effect of corticosteroids on bone is time and dose dependent: they inhibit bone formation and increase bone resorption. Their effect also depends on the underlying disease. In bronchial asthma the changes in bone turnover markers reflect the effects of corticosteroids, whereas in rheumatoid arthritis their effects are summed to that of the underlying inflammatory disease.

HIV-Related Bone Disease and BMT

A reduction of bone mineral density is common among HIV-infected patients. The osteotoxic effect is exerted not only by the HIV virus, but also by highly active antiretroviral therapy (HAART) [23]. In 1995 Serrano et al. conducted a histomorphometric study on 22 HIV-infected patients, showing a notable reduction of bone formation and an enhanced bone resorption in the analyzed specimens [24]. Other in vitro observations confirmed this finding by showing that some viral proteins can inhibit OB development and function [25]. Furthermore, Gibellini et al. used an osteoblast-derived cell line (HOBIT) and primary human osteoblasts as cellular models, showing that HIV-1 may impair bone mass structure homeostasis by TNFalpha regulated osteoblast apoptosis. Under the experimental conditions, this result suggest that apoptosis is regulated by the interaction between HIV-1 gp120 and cell membrane [26]. HIV is also able to decrease osteoblast activity and increase osteoclast-mediated bone resorption in vivo with a reduction of OC and BAP, and a rise in urine N-telopeptide [27].

Several drugs commonly used in HAART regimens can imbalance the bone remodelling cycle; some protease inhibitors (indinavir, ritonavir, saquinavir, nelfinavir) as well as zidovudine have been demonstrated to increase osteoclast activity [28-30]. Recently Mora et al. found that BAP serum concentrations and NTx urine levels of HAART-treated children were significantly higher than those of age-matched healthy controls [31]. Lastly, Guaraldi et al. demonstrated a reduction of serum NTx in HIV-infected patients treated with alendronate, compared with HIV-infected controls treated with placebo [32].

At the last CROI Brown and McComsey presented a study that confirmed that prior to ART initiation bone resorption activity was high and after 6 to 12 months of treatment bone turnover increased markedly. However the changes in bone resorption are not correlated with systemic concentrations of OPG or RANKL or changes in these markers [33]. All these observation clearly show that HIV-related bone disease is characterized by a high turnover state, with an exaggerated osteoclastic bone resorption and, in contrast, with slowed-down osteoblastic bone formation.

Potential Practical Use of BMT

Bone remodelling markers can provide an estimate of the initial skeletal metabolic situation, and subsequently an evaluation of the response to the therapy.

Several studies have demonstrated a significant alteration in bone remodelling marker levels in response to specific therapies [34]. In particular, bisphosphonates seem to reduce the concentration of bone resorption markers most rapidly both in serum and urine. This begins in the first few days of treatment, reaches a plateau usually after 1 to 3 months that is maintained for the entire duration of the therapy and reverts to pretreatment values after withdrawal [35].

Treatment with oestrogens and raloxifene also determines a reduction in resorption markers, showing a slower response, with a constant decrease that reaches a plateau only after 6 to 12 months [36].

Recombinant human parathyroid hormone is the only anabolic agent currently approved for the treatment of osteoporosis. By directly stimulating bone formation, PTH(1-34) and PTH(1-84) peptides reduce the fracture incidence and promptly increase bone neoformation markers (1 month) that correlate with bone structural improvements [37].

A further consideration comes from the fact that the relationship between risk of fracture and bone mass is widely demonstrated [38], and that the above-mentioned drugs significantly alter bone mass. It seems natural to correlate the reduction in fracture risk with the increase in bone mass obtained after therapy, and consequently with the rate of bone remodelling markers reached. However, although numerous studies have been carried out to address this point, a strong correlation between risk of fracture reduction and both increase in bone mass or modifications in the levels of remodelling markers has not been conclusively demonstrated. Although bone mass is a fundamental factor conditioning skeletal resistance, it is not the only one [39]. For these reasons the use of bone remodelling markers to predict
variations in fracture risk after therapy seems inopportune, or at least excessively superficial.

In conclusion, an important decision faced by clinicians dealing with bone metabolic diseases, namely osteoporosis, is which bone remodelling marker/s should be used. Undoubtedly this choice should fall on markers with the highest possible specificity and lowest variability, of which BAP for bone formation and Dpd or CTx for bone resorption would be valid examples. Considering the imbalance between resorption and neodeposition activities that occurs in osteoporosis, we should encounter an increase in both markers. Due to the evidence for the imbalance in favour of the resorption marker, it seems more convenient to rely on a single marker of bone resorption. If the objective is to monitor therapeutic efficacy, then it seems most rational to use a resorption marker for drugs like oestrogens or bisphosphonates that act principally on osteoclasts, whereas for drugs like PTH-peptides that act principally on osteoblasts, a marker for bone neoformation would be more appropriate. It must be kept in mind that serum NTx also identified a correct response to alendronate therapy in HIV-infected patients [32].

The biological variability may create some limitations in the interpretation of the data. Factors that are not modifiable such as age, sex, menopause, recent fractures and diseases, can be controlled in clinical trials using exclusion criteria but have to be taken into account in clinical practice. Moreover the lack of pivotal quality control programmes for bone turnover markers in many countries and the lack of valid reference ranges leaves many clinicians with uncertainties on how to interpret a given result.

At present, the use of these markers has been confined mostly to research mainly due to the absence of guidelines in clinical practice. Yet there is a growing interest in the possibility to use markers in clinical practice. Recently two studies were performed in a large series of pre-menopausal women to determine the premenopausal range values of the BTM [40, 41]. This may help to avoid over-suppression during an antiresorptive therapy in postmenopausal osteoporosis.

What happens in men remains an open issue: osteoporosis in men is less studied than in women. Few studies concern BTM in men: data on the prediction of fragility fractures by BTM are scant. Currently available data are not sufficient to suggest guidelines for the practical use of the markers in clinical management of osteoporosis in elderly men [42]. Nevertheless it is impossible to ignore the utility of bone turnover markers. Considering that they are both easy to obtain and inexpensive, particularly when compared with other not always mandatory examinations, we conclude that the use of bone turnover markers in our clinical practice is justified. No data exist on the relevance of BTM in the management of HIV-infected patients. Following the considerations drawn for the general population we can suggest that, at least in osteoporotic HIV-patients, BTM be used both to increase the ability of fracture prediction and to monitor the response to anti-resorptive therapy.

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