

Serum markers of bone turnover in the diagnosis of renal osteodystrophy

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Renal osteodystrophy (ROD) is a multifactor disorder of bone remodelling observed in patients with chronic renal failure (CRF) from very early stages (CRF stage 2) and associated with significant morbidity¹.

The remodelling cycle in normal bone lasts from four to eight months and includes several phases: 1) An activation phase in which osteoclasts are mobilized and activated; 2) A resorption phase in which osteoclasts erode the bone surface; 3) A reversal phase that ends resorption and osteoblasts are recruited to the eroded surfaces; 4) Unmineralized matrix (osteoid) synthesised by osteoblasts; and 5) Mineralization of the osteoid tissue. Each of these steps (or several of them) are frequently compromised in uraemic patients^{1,2}.

Bone histology remains the gold standard for the diagnosis of renal osteodystrophy and the distinction between high and low bone turnover disease in these patients, frequently requires invasive and costly methods such as bone histomorphometry (including static and kinetic variables after double tetracycline labelling)^{3,4}.

Even though the bone biopsy has been proved to be a safe procedure and one which is free from major complications (incidence lower than 0.7 %)⁵, it has been less performed over the last decade due to several reasons (high cost, difficult in finding experienced teams and appropriate laboratory facilities, etc.). In our experience, with almost five hundred bone biopsies performed, we report less than 0.3 % of (minor) morbidity.

Over the last few years, the use of new therapeutic approaches to ROD (namely the use of sevelamer, lanthanum carbonate, vitamin D analogs and calcimimetics) induced an increased relevance of bone histomorphometry. In fact, these therapies have to be validated at the bone level, and an eventual compromise of the bone mineralization has to be excluded.

But a bone biopsy gives only a picture of the bone lesions at the time that it is performed. It has limitations in the long-term evaluation of the bone turnover, and as an invasive procedure cannot be repeated frequently. Due to these reasons, in clinical practice, less invasive approaches to the diagnosis of ROD were developed.

In recent years, some biochemical markers of bone turnover have been used and tested in the evaluation of bone remodelling in uraemic patients, and their use individually or in combination with other methods will improve the diagnosis of ROD in these patients.

In addition to these serum markers, the role of β 2-microglobulin and of some local mediators involved in the process of bone cells activation and inhibition (such as cytokines and their inhibitors) will be evaluated.

As discussed below, the best approach to the diagnosis of ROD is the dynamic integration of different non-invasive markers of bone remodelling, and when needed, the performing a bone biopsy.

INVASIVE VERSUS NON-INVASIVE DIAGNOSIS OF RENAL OSTEODYSTROPHY

Apart from a quantitative assessment of the cellular elements directly involved in bone turnover, the evaluation of bone histology provides qualitative information on bone tissue structure

and organization (trabecular and cortical) and also of the intra-trabecular space where erythropoiesis takes place. Bone biopsy also allows the quantification and localization of the aluminium and iron bone deposits⁶.

The spectrum of bone changes observed in the uraemic patient, extend from high remodelling bone disease (frequently known as osteitis fibrosa) to low turnover or adynamic disease. Between these two histological diagnoses there are situations of bone mineralization compromise in variable degrees, as is the case of "mixed bone disease" and osteomalacia.

Osteitis fibrosa or predominant hyperparathyroid bone disease is characterised by a high bone formation rate (BFR) (high extension of the trabecular surfaces presenting simple or double tetracycline labels), an increased number of osteoblasts, osteoclasts and eroded surfaces. A marked increase in the bone marrow fibrosis and osteoid surface is usually present.

Mixed uraemic osteodystrophy - typified by signs of secondary hyperparathyroidism with, in addition, increased volume and thickness of osteoid volume is a high subjective diagnosis and usually difficult to interpret by the clinicians. Normally this diagnosis is found in hyperparathyroid bone associated with a compromise of mineralization (ex: vitamin D deficiency, aluminium overload).

Low turnover osteomalacia is characterised by a low BFR associated with an increase in surface, volume and thickness of osteoid.

Finally, adynamic bone disease is characterised by a defect of bone formation, with a marked decrease in active remodelling sites and a low BFR. The prevalence of adynamic bone disease in dialysis patients has been described, through the last decade, as reaching between 15% and 60% of the patients^{1,6,7}. This wide range can be attributed to a different patient criteria selection, differences in the usage of aluminium

versus non-metal based phosphate binders, inappropriate treatment of tap water, different dialysis membranes, variable usage of vitamin D metabolites, etc.

Adynamic disease appears more frequently in diabetic and elderly patients, with a prevalence of between 30 and 50% of biopsied patients. In most cases, it is of iatrogenic nature, resulting from previous parathyroidectomy surgery or excess calcium and/or vitamin D therapy. Aluminium exposure, although progressively more rare, is still a cause of low turnover bone disease^{6,8}.

Almost ten years ago, aluminium overload was still the most frequent cause of adynamic bone disease in Iberoamerican countries, as was shown by our group in a review of 1209 bone biopsies from symptomatic patients^{6,9}. In fact, at that time, two thirds of the patients from Portugal and Spain with adynamic bone disease had a solochrome stainable bone surface aluminium higher than 25%. In our personal experience from the last 3-5 years (still not published) the prevalence of aluminium deposits in bone has decreased significantly.

The suspicion of aluminium overload may be established by the patient dialysis history (water treatment and type of phosphate binders), by the plasma aluminium levels (that have to be interpreted simultaneously with the iron status by measurement of plasma ferritin¹⁰ and by the presence of a positive desferrioxamine test (increase of plasma aluminium superior to 50 mg/L after administration of 5 mg/Kg of desferrioxamine). In the presence of a significant aluminium exposition a bone biopsy seems indicated at least: 1) prior to parathyroidectomy, (since low turnover bone disease may be precipitated 2) and before starting long term desferrioxamine treatment, which is not devoid of hazards (i.e. deafness, fatal mucormycosis)¹¹.

Besides these indications for an invasive diagnosis, a bone biopsy may have to be performed

to establish the diagnosis of ROD disease in symptomatic patients, when the non-invasive markers discussed below are not enough. After kidney transplantation this invasive diagnosis can be particularly relevant to differentiate ROD from the bone effects of the immunosuppressive drugs.

As already mentioned, a significant increase in bone biopsies performed in uraemic patients has been observed during the last years, as part of research protocols to validate new ROD therapies.

Serum markers of bone turnover

We are still looking for a specific and sensitive serum biochemical test for monitoring bone turnover in uraemia. In fact, the ideal biochemical marker of bone turnover should be unique to bone, reflect total skeletal activity and well correlated with histomorphometric and radiocalcium kinetics results.

Several enzymes and matrix proteins synthesised by osteoblasts, and some protein fragments released after bone matrix breakdown during the resorption process, have been proposed as serum biochemical markers of bone formation (bone-specific alkaline phosphatase - bAP; osteocalcin - OC; procollagen type I carboxy-terminal extension peptide - PICP), and of bone resorption (tartrate-resistant acid phosphatase - TRAP; Type I collagen cross-linked telopeptide - ICTP; Pyridinoline cross-links of collagen - Pyr and Dpyr) (Table I).

Unfortunately, the interpretation of serum or plasma levels of these markers is hindered in CRF by different factors (circadian rhythms, diet, age, sex, menopause, liver function, clearance rates, type of dialysis membrane, ultrafiltration, etc.).

As bone remodelling results from the combi-

Table 1**Biochemical markers of bone turnover in uraemic patients**

1. Bone remodelling
 - a. Intact parathyroid hormone (iPTH)
 - b. "Whole" parathyroid hormone; Cyclase activating PTH (whole PTH)
2. Bone formation
 - a. Total alkaline phosphatase (tAP)
 - b. Bone alkaline phosphatase (bAP)
 - c. Osteocalcin or GLA protein (BGP)
 - d. Procollagen type I carboxy-terminal extension propeptide (PICP)
3. Bone resorption
 - a. Tartrate-resistant acid phosphatase (TRAP)
 - b. Pyridinoline cross-links (Pyr; Dpyr)
 - c. Type I collagen cross-linked telopeptide ICTP
 - d. β 2-microglobulin (β 2M)
4. Cytokines and growth factors involved in bone remodelling
 - a. IL-1, IL-6, IL-11, TNF- α ; TGF- β
 - b. GM-CSF, M-CSF
 - c. Osteoprotegerin (OPG)
 - d. RANK, RANK-L

nation of bone formation and bone resorption, it is particularly difficult to evaluate separately these two processes through the serum levels of some biological markers. In fact, this is the major limitation to the use of intact parathyroid hormone (iPTH) in the evaluation of bone remodelling: we cannot obtain information from the bone formation and bone resorption separately.

Measurement of plasma parathyroid hormone and bone turnover

Parathyroid hormone (PTH) is a molecule of 84 amino acids that controls bone turnover. In addition to the intact molecule, PTH fragments containing carboxy-terminal parts of varying length are also present in the circulation. In 1987 an IRMA assay that measured PTH by "sand-

dwiching" it between two antibodies was developed. This first generation IRMA – PTH assay, known as "intact PTH assay", was intended to be free of PTH interference and became the standard in research and clinical practice^{12,13}.

The development of these (IRMA) assays for intact parathyroid hormone (iPTH), has shown that measurement of iPTH levels is a useful predictor of bone histology and can be used as a non-invasive tool in distinguishing between high-turnover (HTBD) and normal or low-turnover bone disease (N/LTBD) when groups of patients are considered¹⁴.

However, on an individual patient, serum iPTH levels alone are frequently not able to distinguish adynamic bone from hyperparathyroid bone disease, as was demonstrated by Qi *et al*¹⁵. According to the results of this group, the iPTH levels between 65 and 450 pg/ml could not predict the bone turnover in dialysis patients, and an invasive diagnosis was proposed for these patients.

The data from Gerakis *et al.* shows that plasma iPTH values below 65 pg/ml, in patients with end stage renal disease, have a poor positive predictive value (45%) in the diagnosis of adynamic bone disease, but present an excellent negative predictive level, even in patients with significant aluminium bone deposition¹⁶. Concerning the diagnosis of HTBD the same authors obtained a positive predictive value of 97% when a value of iPTH 3.5 times above the upper limit of normal, was considered. This positive predictive value dropped to 78% in the presence of aluminium staining.

Subsequently, Quarles *et al.* reported that iPTH assay still showed clinical evidence of fragment interference by overestimating bone turnover¹⁷.

In 1998, D'Amour *et al.* demonstrated that iPTH assay did not measure only full length 1-84 PTH as is was intended, but also measured a 7-84 PTH fragment¹⁸. One year later, a "second

generation" immunoradiometric two-site sandwich assay was developed, which seems to exclusively detect the biological active 1-84 PTH.

This new assay called "whole" PTH - CAP (Cyclase activating PTH) makes use of an antibody directed to the carboxy-terminal part of PTH and another radiolabeled anti-body directed to the first amino-acids of the amino-terminal part of the molecule.

The second generation PTH assays (Whole PTH-CAP from Scantibodies or Bio-intact PTH from Nichols) apparently do not measure a PTH molecule that lacks the first amino-acids nor those PTH fragments, amino-terminally truncated, which are measured by the first generation intact PTH assays¹⁹.

One of the most relevant amino-terminally truncated PTH fragment seems to be the 7-84 PTH, which seems to operate through a different PTH receptor that does not activate adenylate cyclase (7-84 PTH is also called Cyclase Inactive PTH or CIP)^{20,21}.

The 7-84 PTH fragment is present in the circulation of ESRD patients, but is also produced by the parathyroid glands of normal subjects. This fragment seems to inhibit bone resorption and the activation of osteoclasts and osteoblasts in vitro and in vivo^{22,23}. If these results are confirmed with consistent histomorphometric data, 1-84 PTH and 7-84 PTH should be considered as two hormones with opposing biological actions.

According to the recently published Silverberg *and co.* research, the second generation PTH assays were 24% more accurate in the diagnosis of primary hyperparathyroidism than the iPTH assay²⁴. In CRF, these "whole" PTH assays appear to present with values that are approximately half of those achieved with the "intact" PTH assays²⁵.

Some authors postulated that the ratio of 1-84 PTH / 7-84 PTH is helpful in the diagnosis of

adynamic bone disease²⁶, but these results were not reproduced by others.

In a paediatric population of 33 patients treated with peritoneal dialysis, first and second generation immunometric PTH assays showed a similar predictive value of bone turnover and both were best predictors of bone formation than the ratio 1-84 PTH / 7-84 PTH²⁷.

In a recent review from three published studies focused on the relationship between bone histology and plasma PTH levels, as evaluated by first and second generation IRMA PTH assays, Goodman concluded that both assays are highly correlated and have a similar diagnostic value²⁸.

Other PTH fragments of PTH with structural integrity of the (1-4) region but modified in the (15-20) region were recently described by D'Amour, but the clinical implications of these fragments remain to be defined in the uraemic patient²⁹.

The interpretation and significance of PTH levels, independently of the method of determination, in the individual patient is further complicated by other factors, such as the resistance of skeletal to PTH during the evolution of the chronic renal failure, postulated by Massry³⁰ and demonstrated by our group, in the epiphyseal cartilage growth plate of uraemic rats³¹.

It is clear that, in addition to PTH, other tests or new markers of bone remodelling are needed in order to permit a correct and dynamic non-invasive diagnosis of bone turnover. These biochemical markers have to be compared and combined with PTH plasma values to evaluate their additional interest.

Biochemical markers of bone formation

Until now, six **alkaline phosphatase** isoenzymes, produced by different organs have been

identified: liver, bone, kidney, intestine and placenta and tumoral³²⁻³⁵. Interestingly, one single gene codes for alkaline phosphatase (AP), and the isoforms differ only by post-transcriptional glycosilation³⁶.

Bone AP (bAP) is produced by osteoblasts and osteoblasts precursors, and participates in bone formation and in the mineralization process. This molecule provides a high phosphate concentration at the osteoblastic surface and catalyses the hydrolysis of pyrophosphate (an inhibitor of mineralization, that is reduced through this reaction)³⁷.

The bAP molecule has a molecular weight of 80Kda and as is neither dialyzable nor removed by the kidneys, and its plasma levels are not affected by the renal function³⁸. These characteristics of bAP are particularly relevant in the evaluation of renal osteodystrophy.

Several laborious and time-consuming techniques have been used to select for the osteoblast enzyme, bone specific alkaline phosphatase (bAP) and to enhance the sensitivity of this marker, including heat inactivation, wheat germ lectin or concavalin-A precipitation, inhibition by amino acids and urea, high-performance affinity chromatography and agarose gel electrophoresis. The development of monoclonal antibodies specific for bAP have been used in radioimmunological tests (which measure the mass of bAP) and in immunoenzymatic assays (which measure the activity of the isoenzyme), providing the basis for a more specific index of bone formation³⁹.

In 42 haemodialysis patients, we showed that plasma bAP levels (measured with a new direct immunoradiometric assay - IRMA) were better correlated with bone formation and bone resorption histomorphometric parameters than iPTH or total AP levels⁴⁰. Values of bAP higher than 20 ng/ml had a sensitivity of 100% and a specificity of 100% for the diagnosis of HTBD

and there was an excellent correlation between plasma bAP levels and the bone formation rate (BFR). When these limits of bAP values were associated with iPTH serum levels > 200 pg/ml, the positive predictability value for the diagnosis of HTBD increased from 84% to 94%. These results have been confirmed by other groups not only in HTBD patients⁴¹, but also in the diagnosis of adynamic bone disease (ABD) by Couttenye *et al.*, in the latter case by using the agarose-gel electrophoresis method for detection of low levels of plasma bAP⁴².

If a plasma bAP higher than 20 ng/ml, according to our results⁴⁰, (confirmed by other studies⁴³), formally excludes the presence of a low bone turnover disease, we could not define a plasma bAP concentration predictive of adynamic bone disease. It still needs to be demonstrated that BAP is sensitive enough to distinguish between low versus normal bone turnover, and the results from Fletcher *et al.*⁴³ demonstrated that the non-invasive markers of bone turnover, including bAP, were unable to distinguish between low-turnover bone disease, normal bone and mild osteitis fibrosa.

In pre-dialysis end-stage renal patients, bAP demonstrated a high positive predictive value (PPV) of 89% but only if adynamic and normal bone were taken together as one group⁴⁴. In these pre-dialysis patients the combination of an osteocalcin level of 41 ng/L or less with a bAP serum level of 23 U/L or less increased the PPV in the diagnosis of adynamic bone to 77%⁴⁴.

The dissociation between plasma iPTH and plasma bAP levels, frequently observed in uraemic patients may have several explanations and different causes:

- PTH reflects the parathyroid function and bAP the osteoblasts activity. An increase in plasma PTH is not always indicative of high bone turnover disease;

- The presence of aluminium overload may be responsible for the association of high bAP (due to a direct osteoblast stimulation) with low iPTH (resulted from an inhibition of PTH synthesis and release)^{45,46};
- This year, we published our results on a dialysis population of 140 dialysed patients, confirming that higher serum aluminium levels were associated with a significant decrease in the concordance between plasma bAP and iPTH serum levels⁴⁷.
- After successful kidney transplantation plasma iPTH returns to normal in more than 80% patients, but bAP shows a tendency to increase, probably because of an increase in bone turnover^{48,49}. A similar dissociation can frequently be seen in the "hungry bone syndrome" after parathyroidectomy;
- As already discussed in this review, first generation IRMA iPTH assays could overestimate the concentration of active PTH and also contribute to the dissociation with bAP plasma levels;
- In recent years, an uncoupling between bone formation and bone resorption has been reported with apparently increasing frequency by several researchers, which could explain that high PTH levels could be associated predominantly with osteoclasts activity and with normal or low bAP levels⁵⁰.

In summary, plasma bAP in combination with plasma PTH levels increases the sensitivity and specificity in the diagnosis of bone remodelling in uraemic patients. But the clinician has to remember that several conditions can be responsible for the discordance between the levels of these two biochemical markers. In the individual evaluation of a patient all those conditions have to be considered, and bone histomorphometry appears to still be indispensable in some situations.

Osteocalcin or GLA protein (BGP) repre-

sents one of the most abundant non-collagenous bone proteins, that is also present in dentin and calcified cartilage. It is produced by osteoblasts and odontoblasts under the control of 1.25-OH₂D₃ and has been regarded as a marker of bone formation. The carboxylation of this protein depends on vitamin K, as a cofactor of the reaction, and has a half-life of approximately five minutes. The deficit in vitamin K and the decrease in osteocalcin levels have been associated with a decrease in bone mass and a high fracture risk^{51,52}. Even though the association of bone formation with osteocalcin activity is supported by several results, the physiological role of osteocalcin is still not completely understood. For instance, the knock-out mice for osteocalcin present a significant increase in bone formation and bone density⁵³.

Osteocalcin concentrations have been demonstrated to have a significant positive correlation with osteoblastic surface and bone formation rate, in the normal population and in uraemic patients^{54,55}.

Serum intact osteocalcin seems to reflect the excess of produced protein not integrated in bone matrix, or resulting from the process of bone resorption.

The major problem in determining osteocalcin levels by conventional radio immunoassays is distinguishing intact osteocalcin from inactive fragments that accumulate in renal failure. To overcome this problem, a sandwich immunoassay that only detects the intact molecule of serum osteocalcin was developed⁵⁶.

These new immunoradiometric assays, which use specific monoclonal antibodies to measure only intact osteocalcin may be more accurate, (since they will exclude BGP fragments), but they still measure 3 different molecules of unknown significance: total osteocalcin, carboxylated and decarboxylated osteocalcin.

In uraemic patients circulating intact osteo-

calcin represents less than one third of the total osteocalcin. The remaining two thirds correspond to N-terminal, C-terminal and midregion osteocalcin fragments⁵⁷. The biological role of these fragments that accumulate in chronic renal failure is still not clarified.

Osteocalcin also suffers from poor stability and is removed via the kidneys. After menopause its serum levels increased less than those of bAP, showing lower sensitivity and inferior sensibility in the diagnosis of bone turnover in this osteopathy⁵⁸.

In pre-dialysis end stage renal patients, osteocalcin had a positive predictive value (PPV) in the diagnosis of adynamic of only 47%⁴⁴.

In spite of these limitations, in our experience the plasma osteocalcin concentration showed a good sensitivity in distinguishing between high turn-over bone disease versus normal-low bone turn-over. The values were 555 versus 198 ng/mL, respectively⁵⁹. As seen with other biochemical markers of bone turnover the sensitivity of osteocalcin was much lower in the differentiation between low turn-over bone disease and normal bone remodeling.

In a recent work from Morishita on 62 haemodialysis patients who underwent a bone biopsy, the optimal cut-off value to distinguish adynamic bone disease from a mild lesion was 195 pg/mL of serum iPTH or 30 ng/mL of serum intact osteocalcin and the optimal cut-off value to distinguish between hyperparathyroid bone and a mild lesion was 455 pg/mL of serum iPTH or 50 ng/mL of intact osteocalcin concentration⁶⁰.

Although weaker than bAP and iPTH, the correlations of plasma osteocalcin with bone histomorphometric parameters (especially of bone formation and bone mineralization), in haemodialysis patients were quite good⁵⁹.

Procollagen type I carboxy-terminal extension propeptide (PICP), has a molecular weight of approximately 100.000 Da and has been used

as serum marker of bone formation, as it is produced by osteoblasts. PICP results from the cleavage of a molecule of type I procollagen and is a by-product of collagen synthesis.

Collagen type I is the most abundant protein of bone, accounting for more than 90% of the proteins of bone matrix.

The plasma concentration of PICP are determined through the use of specific monoclonal antibodies^{61,62}.

PICP showed a high correlation with dynamic histomorphometric parameters, but not with static histomorphometric parameters nor with other humeral markers of bone turn-over, in pre-dialysis patients⁶³.

In our experience, we found increased levels of PICP in haemodialysis patients, but we did not observe a significant correlation with any of the histomorphometric parameters examined^{59,63} which is in accordance with the results of Mazaferro *et al*⁶⁴.

In patients already on dialysis, the results are contradictory and difficult to interpret in an individual patient. In a population of 18 patients on haemodialysis, Hamdy *et al.* found inappropriately increased PICP levels in patients with aluminium overload (38% of their patients) whereas AP and osteocalcin levels were decreased, also indicating a relatively low specificity and lack of responsiveness of PICP^{64,65}.

BIOCHEMICAL SERUM MARKERS OF BONE RESORPTION

Tartrate-resistant acid phosphatase (TRAP) has been proposed as a potential marker of bone resorption, because it is produced by the osteoclasts, but the degree of specificity to these cells is still not well defined, and a similar fraction enzyme is produced by other cell types (as we can see, for instance, in hairy cell leu-

kaemia and Gaucher's disease)⁶⁶.

The physiological role of TRAP is still not clear, even though it is recognised that it is directly evolved in bone resorption and that TRAP dephosphorylates osteopontin and sialoprotein (constituents of bone matrix)⁶⁷.

TRAP represents the fraction of acid phosphatase that shows resistance, on its mobility on acrylamide gel, to inhibition by tartrate. This is a characteristic of the bone acid phosphatase that was used in the past to separate it from the other isoenzymes.

Immunoassays for the determination of TRAP serum levels were developed, but clinical data are still few, and the value of serum TRAP levels in haemodialysis remains to be clarified⁶⁸⁻⁷⁰.

Median TRAP serum levels were significantly higher in dialysis patients with secondary hyperparathyroid bone disease in comparison with normal or low turnover bone disease patients⁷¹. Serum TRAP strongly correlated with osteoclasts surface in dialysis patients⁷¹.

Pyridinoline cross-links of collagen exist under two chemical forms: Hydroxylisylpyridinoline (or pyridinoline - Pyr) and Lysylpyridinoline (or deoxypyridinoline - DPyr). Both are present in bone and cartilage (in the former DPyr is the predominant type of pyridinoline)⁷².

The concentrations of Pyr and DPyr have been measured with specific polyclonal and monoclonal antibodies, and measured by Elisa or HPLC in urine and later, in serum⁷³.

These molecules are markers of type I and II collagen breakdown and its urinary excretion level has already been validated as an excellent marker of the bone resorption in several metabolic bone diseases^{72,74,75}.

We have demonstrated, for the first time, that serum Pyr concentrations can be reliably measured with an accurate competitive enzyme immunoassay⁷⁶ in serum from dialysis patients,

and that these patients had markedly increased serum Pyr levels compared with normal individuals⁵⁹. In our 37 haemodialysis patients, the highest values of serum Pyr were found in patients with the highest rate of bone resorption⁵⁹.

Our results confirmed pyridinolines as good markers of bone resorption. In fact, we observed a good correlation between serum Pyr levels and the number of osteoclasts/mm² and the percentage of bone covered by osteoclasts⁵⁹. Serum Pyd was correlated significantly better with bone histology than iPTH or osteocalcin.

After transplantation, the urinary excretion of Pyr and DPyr in normocalcemic patients was more sensitive than iPTH and bAP in the diagnosis of secondary hyperparathyroidism⁷⁷.

In opposition with these results, Bervoets *and col.* could not find a significant difference in deoxypyridinoline serum levels in 84 pre-dialysis uraemic patients, with different bone histological disease⁴⁴.

Type I collagen cross-linked telopeptide ICTP is a collagen derivative that is released during bone resorption, and contains cross-linking molecules (pyridinolines).

Assays for ICTP using a polyclonal antibody have been developed but with disappointing clinical results^{11,75}. Its elimination rate depends on the GFR and its serum levels respond poorly to hormone replacement therapy after menopause⁷⁴.

We did not observe, in our dialysed patients, a significant correlation between serum levels of ICTP with any static or dynamic histomorphometric parameter, suggesting that ICTP is not a sensitive marker of bone metabolism in uraemia⁵⁹. On the contrary, Mazzaferro *et al* found ICTP serum levels correlated to serum AP, bAP, iPTH and with some histomorphometric indices of bone turnover, and point out ICTP as a useful humoral marker of bone turnover in dialysis renal osteodystrophy⁶⁴.

β 2-microglobulin (β 2M) is a polypeptide of a molecular weight of 11.810 daltons that accumulates in the serum of uraemic patients. Since the catabolism of β 2M takes place mainly in the kidney, a progressive increase in its serum level is observed with declining renal function, and very high levels are observed in anuric patients with end-stage renal failure⁷⁸.

Serum β 2M levels correlated with serum tartaric resistant acid phosphatase in postmenopausal women^{79;80}.

Particularly, those patients dialysed with a low flux membrane and with a high vintage on dialysis, present significantly increased β 2M serum levels.

β 2M is the main constituent of amyloid deposits found in osteoarticular structures of haemodialysis patients⁷⁹.

We observed that patients with histological high turnover bone disease had higher serum levels than patients with normal/low turnover bone disease and that serum β 2M levels were correlated with serum markers of bone formation rate, namely osteocalcin and bone alkaline phosphatase⁸¹.

In this prospective study, we also found a significant correlation of β 2M levels with a specific serum marker of bone resorption - serum free pyridinoline - but not with intact PTH⁸¹. Lastly, serum β 2M levels were negatively correlated with osteoid volume in patients with high turnover bone disease⁸¹.

Cytokines and local mediators of bone cells activation

Different cytokines and growth factors are directly involved in the activation and control of the bone turnover cycle^{79,82}.

The action of Interleukin-1 (IL-1) and tumour necrosis factor α (TNF- α) are exerted on res-

ting osteoblasts, which cover the trabecular surface. Secondly, osteoblasts and stroma cells of the bone marrow will participate in pre-osteoclast maturation and in the differentiation of these cells into mature and activated osteoclasts by Interleukin-6 (IL-6), Interleukin-11 (IL-11), granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) secretion.

The balance between these cytokines and their specific inhibitors is impaired in haemodialysis patients⁸³.

To study the role of some cytokines in bone turnover process, we measured the circulating levels of different cytokines and of their specific inhibitors by Elisa, using serum of 17 chronically haemodialyzed patients. Blood was drawn at the same time as the bone biopsy was performed, which allowed us to correlate it with the bone histomorphometry results⁸⁴.

In this study we established the presence of increased levels of Interleukin-1, Interleukin-1 receptor antagonist, Interleukin-6 and Interleukin-6 soluble receptor⁸⁴.

Particularly interesting were the reverse relationships observed, on one hand, between the levels of Interleukin-1 receptor antagonist and the osteoblastic surface and on the other, between the Interleukin-6 receptor / Interleukin-6 ratio (IL6-r/IL6) and the osteoclastic surface in our 17 haemodialysis patients.

These results are consistent with the previously described stimulating effects of Interleukin-1 on osteoblasts, suggesting that high serum levels of Interleukin-1 receptor antagonist would render the skeleton less sensitive to bone resorption stimulation induced by Interleukin-1⁸².

The reverse relationship between the Interleukin-6 receptor / Interleukin-6 ratio and the osteoclastic surface, which we observed in our patients, is consistent with *in vitro* studies which demonstrate the role of Interleukin-6 in promo-

ting osteoclast differentiation and mature osteoclast activation⁸⁵.

Other authors published results consistent with ours, which suggest a participation of Interleukin-6 in stimulating bone resorption, and Interleukin-6 may be an action effector of PTH and of other bone resorption stimulating agents⁸⁵.

In bone sections from dialysis patients, stained with antibodies to human interleukin-1alpha, IL-6, IL-11, TNF-alpha, and TGF-beta, intense staining was observed in fibrotic tissue⁸⁶. Immunoreactive TGF-beta and IL-6 was also detected in osteoblasts and osteocytes, suggesting a role of these cytokines in bone cell activation and modulation⁸⁶.

These findings support the hypothesis that the cytokine / cytokine receptor / receptor antagonist network play a relevant role in the pathogenesis of renal osteodystrophy, and that the serum levels of some of these local mediators might reflect the bone cells' activity.

A new cytokine complex, the osteoprotegerin/osteoprotegerin-ligand (OPG/OPGL), involved in osteoclastogenesis and bone remodelling, was discovered. OPG, is a soluble protein that inhibits osteoclastic activation *in vitro* and bone resorption *in vivo*⁸⁷.

Another relevant cytokine with a direct role in bone remodelling at cell level is the recently identified RANKL (receptor activator of NF- κ B ligand). Osteoblasts, activated stromal cells, activated T cells and synovial fibroblasts express RANKL, which is a type II trans-membrane protein found in the surface of the cells and as a proteolytically released soluble form⁸⁸.

Osteoclast progenitors express on their surface the receptor for RANKL, which has been named RANK (receptor activator of NF- κ B). The binding of RANKL to its RANK receptor results in the differentiation and activation of osteoclasts, from its progenitors, which express this receptor on its surface⁸⁹. On the contrary, OPG can inter-

act with RANKL, as a decoy receptor, and prevent the binding of RANKL to RANK. In these circumstances, osteoclastogenesis activation is inhibited⁸⁹.

From the balance between RANKL and OPG a greater or lesser osteoclastic activation and maturation would result. The several systemic (hormonal) and local (cytokines) stimuli of bone remodelling may exert their actions via this RANKL/RANK/OPG axis.

Coen *and col.* reported that, on average, OPG serum levels were elevated in dialysis patients, and correlated with the bone remodelling (lowest levels were found in adynamic bone disease patients)⁹⁰. Similar results were described in 44 dialysis patients by Avbersek-Luznik *et al*, that found OPG consistently increased in dialysis patients, and 1.2 fold higher in patients with a iPTH higher than 200 pg/mL versus those with a iPTH lower than 200 pg/L⁹¹.

In opposition with these results, Haas *et al.* found that OPG levels were significantly reduced in patients with high turnover bone disease when compared with normal or low turnover patients⁹². In the 26 patients studied by these authors, and submitted to a bone biopsy, OPG associated with iPTH was proposed as a marker for non-invasive diagnosis of renal osteodystrophy⁹².

These conflicting results underline the need for more prospective and correctly designed studies to evaluate the role of OPG as a marker of bone remodelling in clinical practice.

CONCLUSION

From the above discussion, it is evident that we are still looking for the ideal biochemical marker of bone turnover.

Besides plasma iPTH, the immunoassays of human bone alkaline phosphatase for bone formation and of pyridinoline and TRAP for bone

resorption seem to be the most sensitive and specific serum markers of bone turnover. These markers should be combined with serum iPTH levels, with serum β 2M levels and with serum aluminium levels.

The quantification of several cytokines and growth factors involved in bone cell modulation will probably acquire a relevant role in the diagnosis of renal osteodystrophy in the near future.

The evaluation of bone turnover should include a combination of different markers, so that the balance between bone formation and bone resorption can be adequately evaluated.

The combination of serum classical biochemical markers with these new identified cytokines will significantly increase the sensibility and the specificity in the evaluation of the bone turnover in uraemic patients.

Finally, in the individual patient, (and more frequently that we had anticipated some years ago), a bone biopsy is still needed to characterize the bone remodelling and bone structure. A bone biopsy is indicated particularly where previous aluminium overload is suspected, in symptomatic patients with discordant biochemical results, in the bone disease after transplantation and to validate new therapies.

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