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Evaluation of bone remodeling in hemodialysis patients: serum biochemistry, circulating cytokines and bone histomorphometry

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Abstract

Background: **To optimize the noninvasive evaluation of bone remodeling, we evaluated, besides routine serum markers, serum levels of several cytokines involved in bone turnover.**
Methods: **A transiliac bone biopsy was performed in 47 hemodialysis patients. Serum levels of intact parathyroid hormone (iPTH; 1-84), total alkaline phosphatases (tAP), calcium, phosphate and aluminum (Al) were measured. Circulating levels of interleukin-6 (IL-6), IL-1 receptor antagonist (IL-1Ra) and soluble IL-6 receptor (sIL-6r) were determined using ELISA. Circulating IL-1β, IL-6, IL-8, IL-10, IL-12p70 and tumor necrosis factor-alpha (TNF-α) were simultaneously quantified by flow cytometric immunoassay.**
Results: **Patients with low/normal bone formation rate (L/N-BFR) had significantly lower serum iPTH ($p<0.001$) and tAP ($p<0.008$) and significantly higher Al ($p<0.025$) than patients with high BFR. Serum calcium and phosphorus, however, did not differ (p=NS). An iPTH >300 pg/mL in association with tAP >120 U/L showed low sensitivity (58.8%) and low negative predictive value (44.0%) for the diagnosis of high BFR disease. An iPTH <300 pg/mL in association with normal or low tAP, <120 U/L, was associated with low sensitivity (66.7%) but high specificity (97.1%) for the diagnosis of L/N-BFR. Serum IL-1, IL-6, IL-12p70 and TNF-α were positively correlated with BFR, serum IL1-Ra and IL-10 with bone area, and by multiple regression analysis, tAP and IL-6 were independently predictive of BFR.**
Conclusions: **Significant associations were found between several circulating cytokines and bone histomorphometry in dialysis patients. The usefulness of these determinations in the noninvasive evaluation of bone remodeling needs to be confirmed in larger dialysis populations.**

Key words: *Bone biopsy, Cytokines, Histomorphometry, Intact parathyroid hormone, Renal Osteodystrophy, Total alkaline phosphatases*

Introduction

Renal osteodystrophy is a multifactorial disorder of bone remodeling, bone mineralizatyion and/or bone quality (volume and structure). It occurs early during the course of chronic kidney disease (CKD; in stage 3), as part of CKD-related bone and mineral disorder (CKD-MBD) (1, 2). Disturbances of bone and mineral metabolism are particularly marked in patients undergoing renal replacement therapy and after kidney transplant, and have been associated with an increase

in extraskeletal calcifications, morbidity and mortality (3).

The bone biopsy remains the gold standard in the evaluation of bone remodeling and bone mineralization (4). According to the Kidney Disease Improving Global Outcomes (KDIGO) consensus definition, it is proposed that the term *renal osteodystrophy* be used exclusively to define changes in bone morphology (requiring a bone biopsy) observed in patients with CKD (1).

Bone histomorphometry is not used in the majority of CKD patients, although it may be of considerable help in the diagnosis of patients with atypical or unexplained skeletal features. Moreover, it remains of great value for direct measurement of bone turnover and also for research purposes. The evaluation of bone turnover (T), bone mineralization (M) and bone volume (V), provided by bone biopsy, would represent the most useful information for clinical practice (TMV classification), as proposed by the KDIGO recommendations (1). Bone biopsy, an invasive procedure which is not easily accepted by CKD patients in most countries, gives a picture of bone morphology at a given point in time (5). Even though it allows the quantification of numerous static and dynamic histomorphometric parameters, bone histology should always be interpreted by the nephrologist in conjunction with clinical, biochemical and epidemiological data. The noninvasive assessment of bone remodeling using new circulating biomarkers is of particular interest in this respect (6).

Serum parathyroid hormone (PTH) levels have become the preferred surrogate measure of bone disease in CKD patients. However, it is limited by lack of sensitivity in the clinical setting (7, 8), particularly in the diagnosis of low-turnover bone disease (9). Because it had become apparent that the second-generation PTH assays measuring the so-called intact PTH (iPTH) at the same time assessed large fragments of the polypeptide (10), novel third-generation assays have been developed to measure the whole PTH molecule alone. The problem with the third-generation assays is that they still need to be validated with respect to bone histomorphometry (10-12). The currently available correlations between serum PTH and bone histomorphometry have all been obtained with second-generation iPTH assays. In addition to this major methodological problem, the specificity of PTH as an indicator of bone turnover also has been questioned by different authors (4, 7, 13, 14).

In the last few decades, numerous circulating markers of bone turnover have been proposed for the noninvasive evaluation of bone remodeling. They include serum markers of bone formation such as osteocalcin, total alkaline phosphatases (tAP), bone-specific alkaline phosphatase (bAP) and procollagen type I C-terminal peptide (PICP), as well as markers of bone resorption such as pyridinoline, deoxypyridinoline, tartrate-resistant acid phosphatase and procollagen type I C-terminal telopeptide (ICTP) (2, 6, 15, 16).

Some of these humoral markers may be useful in the discrimination between high and normal bone turnover (16-18), but their value remains more questionable for the diagnosis of adynamic bone disease (18, 19). A higher sensitivity and specificity in the noninvasive diagnosis of bone remodeling in CKD patients can be obtained by the combination of several of these markers of bone formation and bone resorption (6, 13, 17). However, a convincing distinction between normal- and low-turnover bone disease is much more difficult to achieve in the individual patient, based on the use of such noninvasive markers (9, 13, 14).

In the last few years, several groups of investigators, including ourselves, have demonstrated a major role for several local and circulating growth factors and cytokines in bone metabolism, in particular for the interaction between osteoclasts and osteoblasts (20, 21).

To optimize the noninvasive evaluation of bone remodeling, we evaluated, in addition to routine serum markers, the circulating levels of a variety of cytokines known to be involved in bone turnover. Abnormalities in several of these factors are frequently observed in CKD patients (22, 23). The goal of the present study was to evaluate the combined assessment of conventional serum markers of bone turnover with more recently discovered local activators and inhibitors such as cytokines and their receptors and antagonists in the noninvasive and sequential evaluation of bone remodeling.

Subjects and methods

The present study was performed in 47 white prevalent hemodialysis patients, 17 women and 30 men, aged 48.2 ± 15.0 years (mean ± SD), and treated with a standard hemodialysis procedure of 3 x 4 hours/week, for a minimum duration of 6 months and a mean duration of 81.0 ± 52.5 months. None of the patients was diabetic, presented active liver disease or had been treated with steroids or bisphosphonates. Thirty-one patients were treated with calcitriol. The primary causes of CKD stage 5 were hypertension (n=12), glomerulonephritis (n=10), pyelonephritis (n=6), polycystic kidneys (n=5), interstitial nephrosis (n=5) and other (n=9). Patients were dialyzed with high-flux polysulphone dialyzer and volumetric monitors. Ultrapure water was used, and endotoxin levels were checked by the chromogenic kinetic limulus amebocyte lysate (LAL) method.

All patients who had undergone a bone biopsy at our institution during a time period of 24 months were included in the study. The bone biopsies were performed based on requests by the treating nephrologists for a precise diagnosis

of the underlying osteopathy. The main reasons to perform a bone biopsy were a suspicion of aluminum-related bone disease or a history of significant aluminum exposure (after a treatment with aluminum-based phosphate binders), the indication of surgical parathyroidectomy (particularly in conjunction with previous or ongoing exposure to heavy metals such as aluminum or iron), unexplained hypercalcemia, severe bone pain and pathological fractures.

Bone biopsies

After informed consent was obtained, a transiliac (5-mm diameter) bone biopsy was performed. Iliac crest bone samples for mineralized bone histology and histomorphometry were fixed with ethanol at room temperature, dehydrated and embedded in methylmethacrylate. To measure not only static but also dynamic parameters of bone formation, biopsies were taken after double tetracycline administration (3 days on, 10 days off, 2 days on). These labels were visualized on unstained 10-µm sections by fluorescence microscopy. Sections of 5 µm were stained using the Goldner method and histomorphometrically analyzed for static parameters. Aluminum deposits on trabecular surface and in cortical bone were quantified after specific aluminon staining. A magnification of ×200 was used.

All histomorphometric measurements were performed on a KS-400 image analysis system, running custom macro programs. To obtain histomorphometric and histodynamic measures, an interactive color video-based image analysis system was used.

Results have been reported according to standardized nomenclature (24). The following variables were measured: bone area (B.Ar), osteoid area (O.Ar), osteoid perimeter (O.Pm), eroded perimeter (E.Pm) and quiescent perimeter (Q.Pm). Double-labeled perimeter (dL.Pm) and total perimeter (tL. Pm) were measured on unstained sections. Per section, 10-15 consecutive fields were analyzed. Out of these primary measurements, the following derived parameters were calculated: bone area (B.Ar [%]): the area of trabecular bone including both mineralized bone and osteoid, expressed as a percentage of the total tissue area; osteoid area (O.Ar [%]): the measured area of osteoid, expressed as a percentage of total B.Ar; osteoid perimeter (O.Pm [%]): trabecular bone perimeter occupied by osteoid as a percentage of the total bone perimeter; osteoid width (O.Wi [µm]): the mean width of surface osteoid seams, calculated by dividing the measured osteoid area by the length of the osteoid seams; eroded perimeter (E.Pm [%]): the percentage of trabecular bone perimeter characterized by the presence of scalloped bone resorptive lacunae; double-labeled perimeter (dL.Pm [%]): percentage of total endosteal perimeter exhibiting a double fluorescent tetracycline label; mineral apposition rate (MAR [µm/day]): the rate by which osteoid is mineralized, calculated as the average distance between midpoints of 2 consecutive tetracycline labels, divided by the time interval between the labeling periods (10 days); adjusted apposition rate (AjAr [µm/day]): mineral apposition rate averaged over the entire osteoid perimeter; bone formation rate (BFR [$\mu m^2/mm^2$ per day]): area of bone formed per unit of time, with reference to tissue area, calculated as the product of mineral apposition rate and mineralizing perimeter; the latter is calculated as the sum of doubly labeled plus half of singly labeled perimeter per bone perimeter; osteoid maturation time (Omt [days]): the ratio of osteoid width (O.Wi/MAR, where O.Wi is measured in µm and MAR in µm/day); and mineralization lag time (Mlt [days]): mean interval between deposition and mineralization of any infinitesimal volume of matrix, averaged over the entire life span of the osteoid seam.

Patients were divided into 2 groups according to the accepted criteria for the diagnosis of high bone turnover (High BT) or low/normal bone turnover (Low-Norm BT) (25). A BFR > 613 $\mu m^2/mm^2$ per day and the presence of fibrosis were used to define high-turnover bone disease (25). A BFR between 97 and 613 $\mu m^2/mm^2$ per day was considered normal (25). In 2 patients a diagnosis of normal bone turnover was made in the presence of higher BFR, due to the absence of fibrosis and the normality of all other static and dynamic parameters, underlining the relevance of a global evaluation of bone parameters.

The biopsies were examined and classified by 2 independent observers who had no knowledge of clinical and biochemical findings.

Serum biochemistry

Blood samples were withdrawn at the same time as the bone biopsies, and serum levels of iPTH, tAP, calcium, phosphorus, aluminum and circulating cytokines were evaluated. Serum iPTH was measured with a commercial immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), based on the double antibody technique. The normal iPTH range with this assay is 10-65 pg/mL.

Serum phosphorus and total calcium were also determined by standard automated techniques, with a normal range of 3.0-4.5 mg/dL and 8.8-10.5 mg/dL, respectively. Serum tAP was measured by a standard automated technique as well. Normal values were in the range of 35-120 U/L. Serum levels of aluminum were evaluated by electrothermal atomic absorption spectrometry with Zeeman background correction.

Serum circulating levels of interleukin-6 (IL-6), interleukin-1 receptor antagonist (IL-1Ra), and soluble interleukin-6 receptor (sIL-6r) were determined by ELISA (Medgenix). Serum interleukin-1β (IL-1β), IL-6, interleukin-8 (IL-8), interleukin-10 (IL-10); interleukin-12p70 (IL-12p70) and tumor necrosis factor-alpha (TNF-α) were simultaneously quantified by flow cytometry using Becton Dickinson Cytometric Bead Array (BD-CBA) methodology. This flow cytometric immunoassay employs a series of particles (beads) with discrete fluorescence intensities to simultaneously detect multiple soluble analytes such as cytokines. Each bead in the BD-CBA kit provides a capture surface for a specific cytokine. The mixture of BD-CBA beads is in suspension in order to quantify the analytes by flow cytometry. The system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure, in a small sample volume, soluble analytes in a particle-based immunoassay. We used the BD Human Inflammation CBA kit to measure the above-mentioned cytokine levels in a single sample. The beads with distinct fluorescence intensities coated with specific capture antibodies were mixed together and detected in the FL3 channel of the flow cytometer. This mixture (50 µl/tube) was incubated with diluted recombinant standards (50 µl/tube) as test samples (50 µl/tube). After 1.5 hours of incubation (protected from light), the samples were washed with 1 mL wash buffer and centrifuged. The residual volume of 100 µL was incubated for 1.5 hours protected from light with phycoerythrin (PE)-conjugated antibodies (50 µl/tube). The tubes were again washed with 1 mL of wash buffer and centrifuged. Wash buffer (300 µL) was added to each tube. The samples were acquired with Cell Quest Software in a BD FACS Scan. The flow cytometer setup was previously completed with FACSComp software in Lyse/No Wash mode with BD Calibrite beads. The cytometer setup for the BD-CBA was performed using Cytometer Setup Beads, an FITC Positive Control Detector and a PE Positive Control Detector to adjust the settings for side light scatter (SSC), forward light scatter (FSC), threshold, FL1 PMT, FL2 PMT, FL3 PMT and compensation settings. The number of events acquired was 1,800 to ensure that the sample file contained approximately 300 events per capture bead. Normal levels and limits of detection for cytokines in this cytometric bead array were determined in 8 volunteers (26). The analysis of BD-CBA data was optimized using the BD-CBA software, which uses several matrices to define the standard curves and quantitative results for each cytokine.

Statistical analysis

In descriptive analysis, variables were expressed as median values with range for continuous parameters and frequencies with percentages for categorical parameters; and 95% confidence intervals were presented. Comparisons between values were made using the Mann-Whitney test. Correlations between parameters were evaluated using the Spearman correlation. Further, multiple linear regression was performed considering in the first block the classical clinical parameters that were correlated with BFR, and adding in a second block the cytokines which were also correlated. To perform this analysis, variables that were not normally distributed were submitted to a normalization process through logarithm application. The method used for variable selection when modeling BFR was the stepwise method.
For all analyses performed, a p value of <0.05 was considered as statistically significant. Statistical analysis was performed using SPSS 15.0 for Windows statistical software.

Results

As pointed out above, the 47 patients of the study were classified according to their BT, based on BFR. Eleven patients (23%) were allocated to the Low-Norm BT group (6 patients presented normal bone and 5 adynamic bone disease), and 36 patients (77%) to the High BT group.
Biochemical parameters are presented in Table I. The Low-Norm BT patients had lower serum iPTH and tAP, but higher serum aluminum levels than the High BT patients. In fact, the need to exclude aluminum overload in mineralization front before parathyroidectomy was one of the most frequent reasons for a bone biopsy, as mentioned in the "Methods" section. Serum calcium and phosphorus levels were similar in the 2 groups. Seven patients had aluminum deposits in more than 10% of the trabecular surface (5 in Low-Norm BT group and 2 in High BT group), but only 2 of them (1 in each group) in more than 50% of the trabecular surface.
An increase in iPTH (>300 pg/mL) together with a high tAP value (>120 U/L) showed a very low sensitivity (58.8%) and low negative predictive value (44.0%) for the diagnosis of high-turnover bone disease. An iPTH level below 300 pg/mL in association with normal or low serum tAP (<120 U/L) was associated with a low sensitivity (66.7%) but high specificity (97.1%) for the diagnosis of Low-Norm BT.
Bone histomorphometric and histodynamic data are presented in Table II. High BT patients had significantly higher osteoid area and osteoid perimeter than Low-Norm BT patients, reflecting an increase in bone formation, but without significant impairment of mineralization. Osteoid width was also increased in this group, but clearly below the osteomalacia threshold. High resorption activity was illustrated by a marked increase in eroded perimeter, as observed in the High BT patients. In addition to BFR, the other dynamic

TABLE I
BIOCHEMISTRY DATA ACCORDING TO BONE TURNOVER GROUP

Serum parameters	Low-normal bone turnover group (n=11)	High bone turnover group (n=36)	p Value
iPTH, pg/mL	38 (1-965)	1,077 (215-2,547)	<0.001
Ca, mg/dL	10.5 (8.4-12.6)	9.7 (7.9-11.2)	NS
P, mg/dL	5.8 (1.8-10.7)	6.3 (0-9.8)	NS
Al, μg/L	53.5 (36-148)	35 (0-98)	0.025
tAP, U/L	56 (45-588)	155 (41-1,967)	<0.008

All values are expressed as median (range).
Al = serum aluminum; Ca = serum calcium; iPTH = intact parathyroid hormone; NS = not significant; P = serum phosphorus; tAP = total alkaline phosphatases.

TABLE II
BONE HISTOMORPHOMETRY DATA ACCORDING TO BONE TURNOVER GROUP

Bone parameters	Low-normal bone turnover group (n=11)	High bone turnover group (n=36)	p Value
B.Ar, %	18.7 (12.4-25.2)	20.2 (10.2-45.4)	NS
O.Ar, %	1.2 (0.2-38.2)	5.0 (0.5-27.8)	<0.001
O.Wi, µm	6.3 (4-47.0)	8.9 (5.0-26.1)	<0.001
O.Pm, %	17.8 (2.9-90.7)	38.6 (7.1-72.9)	0.001
E.Pm, %	2.0 (0-4.9)	11.5 (4.5-27.5)	<0.001
dL.Pm, %	6.2 (1.4-16.7)	23.0 (8.5-44.7)	<0.001
MAR, µm/day	1.5 (0.6-2.0)	1.9 (1.1-4.2)	0.03
AjAr, µm/day	0.7 (0.4-1.5)	2.0 (0.5-10.1)	<0.001
BFR, $\mu m^2/mm^2$ per day	245 (69.1-763.6)	1750 (792.0-6420.7)	<0.001
Mlt, days	6.9 (4.3-20.2)	5.4 (0.5-32.0)	NS
Omt, days	4.2 (2.3-11.6)	4.5 (1.6-12.6)	NS

All values are expressed as median (range).
B.Ar = bone area; O.Ar = osteoid area; O.Wi = osteoid width; O.Pm = osteoid perimeter; E.Pm = eroded perimeter; dL.Pm = double-labeled perimeter; MAR = mineral apposition rate; AjAr = adjusted apposition rate; BFR = bone formation rate; Mlt = mineralization lag time; Omt = osteoid maturation time; NS = not significant.

histomorphometric parameters were also significantly increased in High BT group. In contrast, mineralization lag time and osteoid maturation time were normal and similar in the 2 groups, excluding the presence of a significant mineralization defect in these patients.

Serum cytokine levels are presented in Table III, split into 2 groups according to the rate of bone turnover. Serum IL-1β, IL-1Ra, IL-6, sIL-6r, IL-8, IL-10, IL-12p70 and TNF-α concentrations did not differ. Serum calcium, aluminum, IL-10 and IL-1Ra correlated negatively with bone area (B.Ar). Serum tAP correlated positively with osteoid area (O.Ar), osteoid perimeter (O.Pm), BFR and mineralization lag time (Mlt). Serum iPTH also presented highly significant correlations with static (O.Pm and E.Pm) and dynamic (BFR) parameters of bone formation and bone resorption (Tab. IV).

Concerning the relation between BFR and routine serum parameters, iPTH and tAP were the only parameters that showed a positive correlation (ρ=0.55, p<0.001; and ρ=0.51, p=0.001, respectively) (Tab. V). These parameters contributed in a statistical manner to explain BFR changes, according to multiple linear regression coefficients (Tab. VI). When adding to the model the cytokine parameters that were correlated with BFR, namely IL-6, IL-12p70, TNF-α and IL-1beta, the model estimated to explain BFR variations included only tAP and IL-6 (Tab. VI).

Discussion

Despite considerable recent advances in our knowledge of mineral metabolism disturbances and therapeutic modalities, CKD-MBD with its skeletal expression of renal osteodystrophy remains 1 of the most disturbing complications in chronic dialysis patients, and also in kidney transplant recipients.

Bone histology remains the gold standard for the diagnosis of renal osteodystrophy. A clear distinction between

TABLE III
SERUM CYTOKINE LEVELS ACCORDING TO BONE TURNOVER GROUP

Serum parameters	Low-normal bone turnover group (n=7)	High bone turnover group (n=33)	p Value	Normal level (26) (pg/mL)	Limits of detection (pg/mL)
IL-1β, pg/mL	9.6 (0-27.5)	8.6 (0-810)	NS	<48	7.2-5,000
IL-1Ra, pg/mL	541 (184-935)	259 (16-2322)	NS	<260	8-5,000
IL-6, pg/mL	3.3 (0-51.7)	2.0 (0-68.8)	NS	<5.6	3-5,000
sIL-6r, pg/mL	76.5 (57.0-113)	74 (42.0-428)	NS	<80	16-5,000
IL-8, pg/mL	17.5 (9.7-29.4)	16.3 (6.4-69.6)	NS	<40	3.6-5,000
IL-10, pg/mL	3.8 (0-13.8)	1.5 (0-20.4)	NS	<3.7	2.8-5,000
IL-12p70, pg/mL	2.6 (0-8.0)	4.0 (0-172.4)	NS	<9.4	1.9-5,000
TNF-α, pg/mL	0.9 (0-4.5)	2.0 (0-26.0)	NS	<3.7	2.8-5,000

All values are expressed as median (range). Normal levels and limits of detection according to reference (26).
IL-1β = serum interleukin-1β; IL-1Ra = interleukin-1 receptor antagonist; IL-6 = interleukin-6; sIL-6r = soluble interleukin-6 receptor; IL-8 = interleukin-8; IL-10 = interleukin-10; IL-12p70 = interleukin-12p70; TNF-α = tumor necrosis factor-alpha; NS = not significant.

TABLE IV
SIGNIFICANT CORRELATIONS BETWEEN SERUM BIOCHEMISTRY PARAMETERS AND BONE HISTOMORPHOMETRY DATA

Parameters	Ca (mg/dL)	P (mg/dL)	Al (µg/L)	tAP (U/L)	iPTH (pg/mL)	IL-10 (pg/mL)	IL-1Ra (pg/mL)
B.Ar, %	-0.3 (<0.025)		-0.4 (<0.004)			-0.4 (<0.005)	-0.4 (<0.031)
O.Ar, %	-0.3 (<0.036)	-0.3 (<0.025)		+0.7 (<0.001)			
O.Pm, %		-0.4 (<0.009)		+0.6 (<0.001)	+0.4 (<0.003)		
E.Pm, %					+0.6 (<0.001)		
Mlt, days				+0.3 (<0.048)			

Al = serum aluminum; B.Ar = bone area; Ca = serum calcium; E.Pm = eroded perimeter; iPTH = intact parathyroid hormone; Mlt = mineralization lag time; NS = not significant; O.Ar = osteoid area; O.Pm = osteoid perimeter; P = serum phosphorus; tAP = total alkaline phosphatases.

TABLE V
CORRELATIONS WITH BONE FORMATION RATE (BFR)

Serum parameters	ρ (correlation)	p Value
Ca (mg/dL)	-0.165	0.162
P (mg/dL)	0.087	0.301
Al (μg/L(	-0.251	0.064
iPTH (pg/mL)	0.550	<0.001*
tAP (U/L)	0.505	0.001*
IL-6 (pg/mL)	0.334	0.038*
sIL-6r (pg/mL)	0.254	0.092
IL-Ra (pg/mL)	-0.079	0.341
IL-12p70 (pg/mL)	0.358	0.028*
TNF alfa (pg/mL)	0.331	0.040*
IL-10 (pg/mL)	-0.095	0.312
IL-1β (pg/mL)	0.332	0.039*
IL-8 (pg/mL)	0.130	0.251

Ca = calcium; P = phosphorus; Al = aluminum; iPTH = intact parathyroid hormone; tAP = total alkaline phosphatases; IL-6 = interleukin-6; sIL-6r = soluble interleukin-6 receptor; IL-1Ra = interleukin-1 receptor antagonist; IL-12p70 = interleukin-12p70; TNF-α = tumor necrosis factor-alpha; IL-10 = interleukin-10; IL-1β = serum interleukin-1β; and IL-8 = interleukin-8.
*Statistically significant.

high, normal and low bone turnover disease, and a precise evaluation of bone mineralization in uremic patients still requires the invasive and expensive technique of bone histomorphometry, which includes the assessment of static and kinetic variables after double tetracycline labeling (1, 27). Precise knowledge of the underlying bone disease would allow, at least theoretically, a more appropriate treatment approach than that based solely on presently available serum markers of bone turnover (5).

In recent years, a variety of circulating biochemical markers of bone turnover have been tested and used with the purpose of evaluating bone remodeling in uremic patients in the absence of bone biopsy. Used individually or in association with other methods, these biomarkers should allow the nephrologist to improve the diagnosis of renal osteodystrophy in CKD patients (6, 14, 15). This is all the more necessary since the number of centers with expertise in bone histomorphometry has progressively decreased. Moreover, the information provided by bone biopsy (which gives a snapshot of bone lesions) may be significantly increased by the combination with bone remodeling serum marker levels.

Recently, we have shown in a population of 119 hemodialysis patients treated according to the Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines and submitted to a bone biopsy, that adynamic bone disease was the most frequent abnormality (59%). Interestingly, serum iPTH was in the normal or high K/DOQI guideline range in more than one third of the patients with low bone turnover (28). A burning question in clinical practice therefore is how the precise diagnosis of a given type of renal osteodystro-

TABLE VI
MULTIPLE LINEAR REGRESSION COEFFICIENTS

Model 1	Estimated coefficients	95% confidence interval	p Value
iPTH (pg/mL)	0.794	(0.289-1.299)	0.003
tAP (U/L)	1.366	(0.339-2.393)	0.011
Model 2			
tAP (U/L)	1.622	(0.987-2.256)	<0.001
IL-6 (pg/mL)	1.377	(0.253-2.501)	0.018

iPTH = intact parathyroid hormone; tAP = total alkaline phosphatases; IL-6 = interleukin-6.

phy can be reached without performing a bone biopsy, yet providing the patient with the best possible treatment to maintain optimal bone structure and function.

The normal remodeling cycle requires that bone resorption and bone formation take place in a synchronized way, which in turn depends on the systematic development and activation of osteoclasts and osteoblasts, respectively. Osteoclasts originate from hematopoietic granulocyte-macrophage colony-forming units, whereas osteoblasts originate from pluripotential mesenchymal stem cells (29).

Recently, there has been a remarkable improvement in our understanding of the complex biology of bone, with the demonstration of the important role played by a multitude of local and circulating growth factors and cytokines (30). The results of a small number of studies support the hypothesis that the cytokine/cytokine-receptor/receptor antagonist network plays a major role in the pathogenesis of renal osteodystrophy (31, 32), and that the serum levels of some of these local mediators might well reflect bone cell activity (33-36).

Activation of the bone remodeling cycle (activation of osteoblasts) appears to be influenced by high levels of IL-1, such as the ones reported in dialysis patients (37). Similarly, high levels of IL-1 receptor antagonist, which are thought to oppose the effects of IL-1, have been detected in these patients by our group (20). We found an inverse relationship between IL-1 receptor antagonist and osteoblast surface, suggesting that a high serum concentration of the IL-1 receptor antagonist makes the skeleton relatively insensitive to circulating IL-1. On the other hand, at low serum concentration, the IL-1 receptor antagonist would allow the cytokine to fully exert itself in the stimulation of bone formation. In the present study, a negative correlation between IL-1Ra and bone area (B.Ar) was found, in accord with our previous results and the role of this cytokine in osteoblast activation.

Elevated serum levels of IL-6 have also been reported in dialysis patients, as well as in predialysis patients. Montalban et al found a correlation between serum IL-6 and bone remodeling markers in patients with renal osteodystrophy (38). The presence of high serum levels of IL-6 and IL-6 soluble receptor has been repeatedly observed in chronic hemodialysis patients, and our group has reported an inverse relationship between the soluble IL-6 receptor to IL-6 ratio and osteoclast surface (20). These findings are in line with the role of IL-6 in the promotion of osteoclast differentiation, development and activity, as reported by several research groups during the last few years, both in studies performed in vitro and those performed in vivo (21, 38-41). Some of these effects seem to be mediated through the osteoprotegerin/receptor activator of nuclear factor-kB (RANK) ligand/RANK system (40). Our present results also show that IL-6 serum levels are positively correlated with BFR.

Interestingly, in multiple linear regression analysis, IL-6 serum levels were independently predictive of BFR. In our opinion, these results are particularly relevant, as IL-6 is a highly stable cytokine, with recognized systemic effects (38, 40).

In the present study, serum IL-10 levels correlated negatively with bone area (B.Ar). IL-10 is produced by monocytes, macrophages, B cells and T cells. It inhibits the production of several proinflammatory cytokines, including IL-1, and thereby interferes with osteoblast stimulation (42). This action could be the mechanism underlying the observed reduction in bone area, but this hypothesis requires further investigation.

IL-12 is a proinflammatory cytokine produced by monocytes/macrophages, B cells and dendritic cells. It enhances myelopoiesis of primitive bone marrow progenitor cells and

synergizes other cytokines to stimulate proliferation of resting cells. Its effects on bone turnover still need to be clarified, but due to its effects on the monocyte/macrophage lineage, it probably also affects osteoclast proliferation and maturation (43, 44).

Our study presents several limitations, including a relatively high aluminum exposition of the cohort evaluated, the use of only second-generation intact PTH levels and the absence of serum vitamin D sterol measurements. The results of our study also need to be confirmed in larger patient populations, with a better balance between cases having high and low bone turnover bone. Interestingly a significant mineralization defect was not observed in any of our patients, excluding osteomalacia associated with aluminum intoxication or severe vitamin D deficiency.

In conclusion, we show that serum IL-1, IL-6, IL-12p70 and TNF-α are positively correlated with BFR, serum IL1-Ra and IL-10 with bone area and that serum IL6 is an independent predictor of BFR, in a population of prevalent dialysis patients. The quantification of the serum levels of these cytokines through a simple and reproducible flow cytometry method may be relevant for research purposes or to complement the classical biochemical markers of bone turnover (such as iPTH and tAP). The usefulness of these serum cytokine determinations in the noninvasive evaluation of bone remodeling needs to be confirmed in larger dialysis populations.

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References

1. Moe SM, Drüeke T, Cunningham J, et al. Definition, evaluation and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int. 2006;69:1945-1953.
2. Coen G, Mazzaferro S, Ballanti P, et al. Renal bone disease in 76 patients with varying degrees of predialysis chronic renal failure: a cross sectional study. Nephrol Dial Transplant. 1996;11:813-819.
3. Martin KJ, Olgaard K, Coburn JW, et al. Diagnosis, assessment and treatment of bone turnover abnormalities in renal osteodystrophy. Am J Kidney Dis. 2004;43:558-565.
4. Ferreira A. Diagnosis of renal osteodystrophy: when and how to use biochemical markers and non-invasive methods; when one biopsy is needed. Nephrol Dial Transplant. 2000;15:8-14.
5. Ho LT, Sprague S. Percutaneous bone biopsy in the diagnosis of renal osteodystrophy. Semin Nephrol. 2002;22:268-275.
6. Ferreira A, Drüeke TB. How to diagnose the different forms of renal osteodystrophy: role of biochemical markers. Am J Med Sci. 2000;320:85-89.

7. David V, Lafage-Proust MH, Laroche N, Christian A, Ruegsegger P, Vico L. Two-week longitudinal survey of bone architecture alteration in the hindlimb-unloaded rat model of bone loss: sex differences. Am J Physiol Endocrinol Metab. 2006;290:440-447.
8. Qi Q, Maunier-Faugere MC, Geng Z, Malluche H. Predictive value of serum parathyroid hormone levels for bone turnover in patients on chronic maintenance dialysis. Am J Kidney Dis. 1995;22:622-631.
9. Cohen-Solal ME, Sebert JL, Boudailliez B, et al. Comparison of intact, midregion and carboy terminal assays of parathyroid hormone for the diagnosis of bone disease in hemodialyzed patients. J Clin Endocrinol Metab. 1991;71:516-524.
10. Barreto FC, Barreto DV, Moysés RMA, et al. K/DOQI-recommended intact PTH levels do not prevent low-turnover bone disease in hemodialysis patients. Kidney Int. 2008;73:771-777.
11. Lepage R, Ray L, Bossard J-H, et al. A non-(1-84) circulating parathyroid hormone (PTH) fragment interferes significantly with intact PTH commercial assay measurements in uremic samples. Clin Chem. 1998;44:805-809.
12. Malluche H, Mawad H, Trueba D, Monier-Faugere M-C. Parathyroid hormone assays: evolution and revolutions in the care of dialysis patients. Clin Nephrol. 2003;59:313-318.
13. Nguyen-Yamamoto L, Rousseau L, Brossard JH, Lepage R, D'Amour P. Synthetic carboxy-terminal fragments of parathyroid hormone (PTH) decrease ionized calcium concentration in rats by acting on a receptor different from PTH/PTH related peptide receptor. Endocrinology. 2001;142:1386-1392.
14. Coen G, Ballanti P, Bonnuci E, et al. Bone markers in the diagnosis of low turnover osteodystrophy in haemodialysis patients. Nephrol Dial Transplant. 1998;13:2294-2302.
15. Goodman WG, Salusky IB, Jüpner H. New lessons from old assays (PTH), its receptors, and the potential biological relevance of PTH fragments. Nephrol Dial Transplant. 2002;17:1731-1736.
16. Ureña P, de Vernejoul MC. Circulating biochemical markers of bone remodeling in uremic patients. Kidney Int. 1999;55:2141-2156.
17. Ureña P, Ferreira A, Kung V, et al. Serum pyridinoline as a specific marker of collagen breakdown and bone metabolism in hemodialysis patients. J Bone Miner Res. 1995;10:932-939.
18. Bervoets AR, Spasovski GB, Behets GJ, et al. Useful biochemical markers for diagnosing renal osteodystrophy in predialysis end-stage renal failure patients. Am J Kidney Dis. 2003;41:997-1007.
19. Ureña P, Hruby M, Ferreira A, Ang KS, de Vernejoul MC. Plasma total versus bone alkaline phosphatase as markers of bone turnover in hemodialysis patients. J Am Soc Nephrol. 1996;7:1-7.
20. Couttenye MM, D'Haese PC, Van Hoof VO, et al. Low serum levels of alkaline phosphatase of bone origin: a good marker of adynamic bone disease in haemodialysis patients. Nephrol Dial Transplant. 1996;11:1065-1072.
21. Ferreira A, Simon P, Drüeke T, Descamps-Latscha B. Potential role of cytokines in renal osteodystrophy. Nephrol Dial Transplant. 1996;11:399-400.
22. Duarte ME, Carvalho EF, Cruz EA, Lucena SB, Andress DL. Cytokine accumulation in osteitis fibrosa of renal osteodystrophy. Braz J Med Biol Res. 2002;35:25-29.
23. Herbelin A, Nguyen AT, Zingraff J, Ureña P, Descamps-Latscha B. Influence of uremia and hemodialysis on circulating interleukin-1 and tumor necrosis factor alpha. Kidney Int. 1990;37:116-125.
24. Herbelin A, Ureña P, Nguyen AT. Elevated circulating levels of interleukin-6 in patients with chronic renal failure. Kidney Int. 1991;39:954-960.
25. Parfitt AM, Drezner MK, Glorieux FH, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. J Bone Miner Res. 1987;6:595-610.
26. Salusky IB, Coburn JW, Brill J, et al. Bone disease in pediatric patients undergoing dialysis with CAPD or CCPD. Kidney Int. 1988;:38975-38982.
27. Suntharalingam G, Perry MR, Ward S, et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med. 2006;355:2591-2592.
28. Ferreira A. Development of renal bone disease. Eur J Clin Invest. 2006;36:2-12.
29. Ferreira A, Frazão JM, Monier-Faugere M-C, et al. Effects of sevelamer hydrochloride and calcium carbonate on renal osteodystrophy in hemodialysis patients. J Am Soc Nephrol. 2008;19:405-412.
30. Boyle WB, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423:337-342.
31. Hruska KA, Teitelbaum SL. Mechanisms of disease: renal osteodystrophy. N Engl J Med. 1995;333:166-174.
32. Jung Y, Wang J, Schneider A, et al. Regulation of SDF-1 (CXCL12) production by osteoblasts: a possible mechanism for stem cell homing. Bone. 2006;38:497-508.
33. Hruska KA. New concepts in renal osteodystrophy. Nephrol Dial Transplant. 1998;13:2755-2760.
34. Koh AJ, Demiralp B, Neiva KG, et al. Cells of the osteoclast lineage as mediators of the anabolic actions of parathyroid hormone in bone. Endocrinology. 2005;146:4584-4596.
35. Ferreira A, Ghazali A, Galvão J, et al. Effect of type of dialysis membrane on bone in haemodialysis patients. Nephrol Dial Transplant. 2001;16:1230-1238.
36. Steddon SJ, McIntyre CW, Schroeder NJ, Burrin JM, Cunningham J. Impaired release of interleukin-6 from human osteoblastic cells in the uremic milieu. Nephrol Dial Transplant. 2004;19:3078-3083.
37. Barreto FC, Barreto DV, Moyses RM, et al. Osteoporosis in hemodialysis patients revisited by bone histomor-

phometry: a new insight into an old problem. Kidney Int. 2006;69:1852-1857.
38. Descamps-Latscha B, Herbelin A, Nguyen AT, et al. Balance between IL-1 beta, TNF alpha, and their specific inhibitors in chronic renal failure and maintenance dialysis: relationships with activation markers of T cells, B cells and monocytes. J Immunol. 1995;154:882-892.
39. Montalban C, Garcia-Unzueta MT, De Francisco A, Amado JA. Serum interleukin-6 in renal osteodystrophy: relationship with serum PTH and bone remodeling markers. Horm Metab Res. 1999;31:14-17.
40. Santos FR, Moyses RM, Montenegro FL, Jorgetti V, Noronha IL. IL-1beta, TNF-alpha, TGF-beta and BFGF expression in bone biopsies before and after parathyroidectomy. Kidney Int. 2003;63:899-907.
41. Liu XH, Kirschenbaum A, Yao S, Levine AC. Cross-talk between the interleukin-6 and prostaglandin E(2) signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of nuclear factor-kB (RANK) ligand / RANK system. Endocrinology. 2005;146:1991-1998.
42. Kudo O, Sabokbar A, Pocock A, et al. Interleukin-6 and interleukin 11 support human osteoclast formation by a RANKL-independent mechanism. Bone. 2003;32:1-7.
43. Ernest HS, Choy MD, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med. 2001;344:907-916.
44. Jacobsen SE, Veyby OP, Smelan EB. Cytotoxic lymphocyte maturation factor (interleukin 12) is a synergistic growth factor for hematopoietic stem cells. J Exp Med. 1993;178:413-418.
45. Kollet O. Ayelet Dar, Lapidot T. The multiple roles of osteoclast in host defense: bone remodeling and hematopoietic stem cell mobilization. Annu Rev Immunol. 2007;25:51-69.

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