Diagnostic accuracy of noninvasive bone turnover markers in renal osteodystrophy

DIAGNOSTIC ACCURACY OF NON-INVASIVE BONE TURNOVER MARKERS IN RENAL OSTEODYSTROPHY

Hanne Skou Jørgensen (MD, PhD)[1,2], Geert Behets (PhD)[3], Liesbeth Viaene(MD, PhD)[4], Bert Bammens (MD, PhD)[1, 5], Kathleen Claes (MD, PhD)[1, 5], Bjorn Meijers (MD, PhD)[1, 5], Maarten Naesens (MD, PhD)[1, 5], Ben Sprangers (MD, PhD)[1, 5], Dirk Kuypers (MD, PhD)[1, 5], Etienne Cavalier(MD, PhD)[6], Patrick D'Haese (PhD)[3], Pieter Evenepoel (MD, PhD)[1, 5]

[1] Department of Microbiology, Immunology and Transplantation; Nephrology and Renal Transplantation Research Group, KU Leuven, Belgium

[2] Department of Kidney Diseases, Aarhus University Hospital, Aarhus, Denmark

[3] Laboratory of Pathophysiology, University of Antwerp, Wilrijk, Belgium

[4] Department of Nephrology, Az Groeninge, Kortrijk, Belgium

[5] Department of Nephrology and Renal Transplantation, University Hospitals Leuven, Belgium

[6] Department of Clinical Chemistry, Université de Liège, Liège, Belgium

Corresponding author

Peter Evenepoel

Division of Nephrology, University Hospitals Leuven,

Herestraat 49, B-3000 Leuven, Belgium.

E-mail: Pieter.Evenepoel@uzleuven.be

Abstract

Rationale & Objective: A bone biopsy remains the gold standard for diagnosing renal osteodystrophy as non-invasive alternatives have yet to be established. The aim of this study was to investigate the diagnostic accuracy of biochemical markers of skeletal remodeling for bone turnover.

Study Design: Cross-sectional retrospective diagnostic test study.

Setting & Participants: Patients with chronic kidney disease stages G4-G5D and kidney transplant recipients with successful transiliac bone biopsies.

Tests Compared: Bone turnover as determined by bone histomorphometry was compared to the following biochemical markers: Full-length (1-84) parathyroid hormone (PTH), bone-specific alkaline phosphatase (BsAP), intact procollagen type I N-terminal propeptide (PINP), and tartrate-resistant acid phosphatase isoform 5b (TRAP5b).

Outcome: Diagnostic performance was evaluated by area under the receiver operator characteristics curve (AUC), sensitivity, specificity, and negative and positive predictive values. Optimal diagnostic cutoffs were determined in an exploration cohort (n=100) and validated in a separate cohort (n=99).

Results: All biomarkers differed across categories of low 33 (17%), normal 109 (55%), and high 57 (29%) bone turnover. AUC values were in the range of 0.75 – 0.85, with numerically higher values for the bone turnover markers. High negative predictive values (≥90%) were found for both high and low bone turnover, indicating the ability to rule out both conditions using the suggested biomarker cutoffs. The highest diagnostic performance were seen with combinations of biomarkers, with overall diagnostic accuracies of 90% for high turnover, and 78% for low turnover. Results were comparable for kidney transplant candidates and recipients in a sensitivity analysis.

Limitations: The single-center approach and heterogeneity of the study cohort are main limitations of this study.

Conclusions: We conclude that the diagnostic performance of biochemical markers of bone turnover is acceptable, with clinical utility in ruling out both high and low turnover bone disease.

Key words

Alkaline phosphatase, Bone histomorphometry, Chronic kidney disease – mineral and bone disorder, Parathyroid hormone, Kidney transplantation, Sensitivity and specificity, Tartrate-Resistant Acid Phosphatase

Non-invasive biomarkers can rule out high and low bone turnover in patients with chronic kidney disease

Skeletal remodeling is disturbed in chronic kidney disease, and knowledge of the bone turnover status may help guide treatment decisions. Currently, a bone biopsy is the diagnostic standard to evaluate bone turnover, but this invasive procedure is not well suited for everyday clinical practice. This study investigates the diagnostic potential of non-invasive, biochemical bone turnover markers for a diagnosis of high or low bone turnover in patients with chronic kidney disease. Results indicate that biomarkers may be used to rule out the presence of both high and low bone turnover with a high degree of certainty. The clinical implications of these findings are that biochemical bone turnover markers may aid treatment decisions and potentially decrease the need for invasive bone biopsies in our patients.

Introduction

The transiliac bone biopsy remains the gold standard for diagnosing renal osteodystrophy.[1] However, due to its invasive nature, a bone biopsy is not recommended as part of routine clinical workup.[2] Rather, it is reserved for the in-depth evaluation of bone disease in select patients, e.g. in cases of skeletal fractures or bone pain, inconsistencies in biochemical parameters of mineral metabolism, prior to referrals for parathyroidectomy, or initiation of anti-resorptive therapy.[3] Suitable alternatives to the bone biopsy for evaluating renal osteodystrophy in everyday clinical practice have yet to be established.

In the most recent Kidney Disease – Improving global outcomes (KDIGO) guidelines on mineral and bone disorder in chronic kidney disease (CKD),[2] circulating levels of parathyroid hormone (PTH) and bone-specific alkaline phosphatase (BsAP) are suggested as surrogate markers of bone turnover in CKD stages 3-5D, with the specification that both may predict bone turnover at markedly high or low levels. As a biomarker, however, PTH has several limitations, including a high biological variability and assay standardization issues.[4] Further, skeletal sensitivity to PTH may be reduced in CKD,[5] and this PTH hyporesponsiveness may depend on kidney function. Despite these limitations, PTH has long been the preferred marker of bone turnover in clinical practice, and novel biomarkers so far failed to show diagnostic superiority, at least in patients with kidney failure.[6–8]

In contrast to PTH, which is a key regulator of bone metabolism, bone turnover markers are specifically released from bone cells during the skeletal remodeling process. BsAP is secreted by osteoblasts in the process of biomineralization,[9] and procollagen type I N-terminal propeptide (PINP) is a fragment released as collagen is deposited in the bone matrix;[10] both are considered markers of bone formation. Importantly, monomeric fragments of PINP accumulate in CKD, necessitating the use of assays specific for the intact, trimeric form.[11] Tartrate-resistant acid phosphatase isoform 5b (TRAP5b) is an enzyme originating from osteoclasts, and considered a highly specific marker of bone resorption.[12] As neither of these biomarkers are influenced by kidney function, they may be particularly well suited for use in patients with CKD.[13]

The primary aim of the present study was to investigate the diagnostic accuracy of a panel of biochemical markers of skeletal remodeling as compared to biointact PTH, using the histomorpometric evaluation of bone

turnover as the diagnostic standard. We hypothesized that biochemical markers of bone turnover would outperform PTH for a diagnosis of high or low bone turnover in patients with CKD.

Materials and methods

Cohort

Patients with CKD and a tetracycline (TC)-labelled bone biopsy of sufficient quality for a full histomorphometric analysis were eligible to be included. All patients had agreed to participate in a prospective, observational study on the natural history of mineral and bone disorder after kidney transplantation (clinical trial identifier: NCT01886950). Use of anti-osteoporotic treatment was the only exclusion criterion. All bone biopsies were performed between April 2011 and Sept 2018 at Leuven University Hospitals (Leuven, Belgium).

Relevant demographic variables and details of medical therapy were extracted from electronic patient files. For kidney transplant recipients, immunosuppression was achieved with glucocorticoids, a calcineurin inhibitor, and an antimetabolite. Methylprednisolone was administered intravenously on the day of transplantation (500 mg) and the first post-operative day (40 mg), followed by a daily dose of 16 mg prednisolone orally, tapered gradually over the first 3 months. The clinical course, together with findings on a protocolled kidney graft biopsy at month 3 determined if glucocorticoids were discontinued.

All clinical and research activities reported are consistent with the Principles of the Declaration of Istanbul on Organ Trafficking and Transplant Tourism. The study adhered to the principles of the Declaration of Helsinki and was approved by the Ethical Committee of KU Leuven (study identifier: S52091). All participating patients provided written informed consent.

Biochemical analyses

Blood samples were taken after an overnight fast, on the day of the bone biopsy procedure. Whole blood samples were stored for less than 2 hours at 5°C before arrival at the laboratory, where they were centrifuged at 3000 rpm for 10 minutes, after which serum samples were aliquoted, and stored at –80°C until analysis. Creatinine, hemoglobin, phosphate, total calcium, and total alkaline phosphatase (AP) were measured using

standard laboratory techniques. The estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation.[14]

Serum 25-hydroxy vitamin D (calcidiol) was measured using a radioimmunoassay.[15] Serum concentrations of full-length, "biointact" PTH was determined by an in-house immunoradiometric assay (normal range 3–40 ρg/mL).[16] Bone-specific alkaline phosphatase (BsAP; normal range 6.1–25.5 ug/L, assay range 1–75 µg/L), trimeric procollagen type I N-terminal propeptide (PINP; normal range 12.8–82.6 ng/mL, assay range 2–230 ng/mL), and tartrate-resistant acid phosphatase isoform 5b (TRAP5b; normal range 1.1–6.9 U/L; assay range 0.9–14.0 U/L) were measured with the ImmunoDiagnostic Systems iSYS instrument (IDS, Boldon, UK). Inter- and intra-assay variation were below 10% for all assays used. Values of BsAP, Intact PINP, and TRAP5b above the upper limit of quantification of the respective assays were determined after dilution.

Bone histomorphometry

The bone biopsy was performed as an outpatient procedure under light sedation with local anesthesia. Using an 8G trephine with an outer/inner diameter of 4.50/3.55 mm (Biopsybell [Mirandola, Italy]), the sample was retrieved from a site 2 cm posterior and 2 cm inferior to the anterior iliac spine. Complications occurred in 7 patients and included prolonged post-procedural pain (n = 6) and bleeding requiring transfusion (n = 1). All biopsies were preceded by double- TC-labeling by the following protocol: TC was administered orally (500 mg x 2 daily for 3 days) for two sessions with a TC-free interval of 11 days, and the bone biopsy was scheduled 4 days after the last intake of TC. To avoid a systematic bias caused by lack of TC labels in very low bone turnover, biopsies without visible TC labels were included if the patient was noted to have taken TC and static histomorphometry was consistent with low turnover. In such cases, the bone formation rate by total tissue volume (BFR/TV) was set arbitrarily at 1 µm³/cm³/year (n = 12).[17] Similarly, patients with a very high bone turnover and diffuse, unmeasurable labels (n = 2) were not excluded, and a value of 712 µm³/cm³/year was set for the BFR, corresponding to 20% above the highest measured BFR in this cohort (594 µm³/cm³/year).

Bone cores were fixed in 70% ethanol and embedded in a methylmethacrylate resin. Un-decalcified 5-µm thick sections were stained by the Goldner method to determine static bone parameters, and unstained 10-µm

thick sections were mounted in 100% glycerol for fluorescence microscopy to visualize tetracycline labels and determine the dynamic parameters. All bone histomorphometric analyses were performed at the Laboratory of Pathophysiology, University of Antwerp, Belgium, running a custom program on the commercially available image analysis software AxioVision (version 4.51, Zeiss Microscopy, Zeiss, Germany). Bone histomorphometric parameters are reported in two dimensions using standardized nomenclature,[18] following the recommended approach of evaluating bone turnover, mineralization, and volume.[1] Patients were categorized as having low, normal, or high bone turnover based on the BFR/TV, using a normal range of 11.5 – 110 mm³/cm³/year, as previously determined in an adult reference population.[19,20]

Statistical analysis

Descriptive statistics are presented as mean ± SD, median [IQR], or *n* (%), as appropriate. Differences in biomarker levels across categories of bone turnover were tested by one-way analysis of variance (ANOVA), followed by Student's *t* test against the "Normal" group, using the natural logarithms of biomarkers to achieve normal distribution. The diagnostic ability of biomarkers were evaluated by area under the receiver operator characteristics curve (AUC) statistics,[21] running a non-parametric and bootstrapped estimation for each biomarker. AUC values were designated as poor (<0.6), fair (0.6–0.7), good (0.7–0.8), very good (0.8–0.9), or excellent (>0.90). Optimal cutoffs were determined by Liu's method,[22] as the biomarker level resulting in the maximum product of sensitivity and specificity. To avoid model optimism, we randomly split the cohort into separate exploration (n = 100) and validation (n = 99) subsets. Estimation of the optimal cutoff of each biomarker was performed in the exploration cohort, and the diagnostic performance of these cutoffs were subsequently evaluated in the validation cohort. To account for the effect of disease prevalence on test performance, we adjusted the negative and positive predictive values for pre-test probability (i.e. prevalence of disease) according to Bayes' theorem.[23] Finally, as a sensitivity analysis, we conducted AUC analyses for patients biopsied before or after kidney transplantation as two separate groups. Statistical analyses were performed using the commercially available STATA IC version 16.1 (StataCorp LP, College Station, TX, USA).

Results

Cohort

Out of 286 available biopsies, 19 were of insufficient quality for a full histomorphometric analysis, and 22 were without successful TC labeling. Further, 35 patients were excluded to avoid duplicates, 4 due to bisphosphonate use, 4 due to recent fracture (<6 months), and 3 due to missing biochemistry, leaving a cohort of 199 patients for analysis (**Figure** S1). The majority of bone biopsies were performed solely as part of a research protocol (n = 187, 95%), while the remaining 12 patients were biopsied on indication.

Table 1 Demographic data in the overall cohort and separated by time of bone biopsy before or after kidney transplantation

	CKD G4-G5D (*n* = 80)	Kidney transplant recipients (*n* = 119)
Age, years	53 ± 14	57 ± 11
Gender, male	49 (61%)	85 (71%)
Diabetes mellitus, any type	8 (10%)	37 (31%)
Previous parathyroidectomy	5 (6%)	16 (14%)
Body mass index, kg/m²	24.4 ± 4.6	25.3 ± 4.8
Bone turnover		
Low	6 (8%)	27 (23%)
Normal	38 (47%)	71 (60%)
High	36 (45%)	21 (18%)
Biointact PTH, ρg/mL	277 [111; 461]	59 [40; 110]
Total AP, U/L	112 [91; 168]	79 [59; 98]
Total calcium, mg/dL	9.46 ± 0.76	9.87 ± 0.79
Phosphate, mg/dL	5.66 ± 1.70	3.09 ± 0.83
Calciciol, ng/mL	38 ± 17	36 ± 14
BsAP, ug/L	37.2 [24.7; 58.3]	21.8 [14.2; 31.7]
Intact PINP, ng/mL	108.2 [61.1; 167.3]	55.0 [30.1; 98.8]
TRAP5b, U/L	6.1 [4.4; 8.3]	3.5 [2.5; 4.9]
Mimpara	6 (8%)	2 (2%)
Vitamin D	55 (69%)	53 (45%)
Active vitamin D	42 (53%)	20 (17%)
Methylprednisolone	18 (23%)	88 (74%)

Data are mean ± SD, median [IQR], or *n* (%)

Abbr.: AP=alkaline phosphatase, BFR=Bone formation rate, BsAP=bone-specific alkaline phosphatase, PINP=pro-collagen type I pro-peptide, PTH=parathyroid hormone, TRAP5b=tartrate resistant acid phosphatase isoform 5b

Demography

Mean age was 55 ± 13 years, two-thirds were men (67%), and 23% had diabetes mellitus. The cause of CKD was as follows: Cystic or hereditary disease (*n* = 49, 25%), glomerulonephritis or vasculitis (*n* = 49, 25%), hypertension or large vessel disease (*n* = 17, 8.5%), diabetic nephropathy (*n* = 15, 7.5%), interstitial nephritis (*n* = 15, 7.5%), miscellaneous (*n* = 7, 3.5%), or unknown (*n* = 47, 24%). Demographic data for participating kidney transplant candidates (*n* = 80) and recipients (*n* = 119) are given in **Table** 1. Kidney transplant candidates were CKD stage G4 (*n* = 7), CKD stage G5 (*n* = 7), or CKD stage G5D (*n* = 66). Among patients with CKD stage G5D, 44 received chronic intermittent hemodialysis therapy and 22 continuous peritoneal dialysis treatment, with a median dialysis vintage of 15 [9, 26] months. Kidney transplant recipients were mainly biopsied at 12 months post-transplant (*n* = 103), with 6 patients biopsied earlier and 10 patients later than this time-point. Transplant recipients had a mean eGFR of 50±15 ml/min/1.73m²; 8 patients had an eGFR <30, and none <15. The immunosuppressive regimen consisted of a calcineurin inhibitor in combination with mycophenolate mofetil (MMF) for the majority (*n* = 110, 92%); 8 patients received a calcineurin inhibitor without MMF, and 1 patient received MMF alone. Tacrolimus was the most common calcineurin inhibitor used, with only two patients receiving cyclosporine A. Prednisolone had been halted in 33 (28%) patients, and for the remaining, daily doses were ≤ 4 mg (*n* = 85), with a single patient receiving 10 mg. The median cumulative steroid dose at 12 months post-transplant was 2.54 [1.81, 3.14] g.

Biochemical markers of bone turnover

Bone turnover was classified as low in 33 (17%), normal in 109 (55%), and high in 57 (29%) patients. Levels of biochemical markers by categories of bone turnover are given in **Table** 2 and **Figure** 1. Highly significant differences in circulating levels were seen for all markers across bone turnover categories, although with a noticeable overlap in biomarker range, particularly between patients with low and normal bone turnover.

Table 2 Biochemical markers by category of bone turnover

	Normal range	Low (n = 33)	Normal (n = 109)	High (n = 57)	p
Biointact PTH, ρg/mL	3–40	53.2 [36.8; 86.5]	77.6 [43.4; 175.0]	249.0 [114.5; 428.6]	<0.001
Biointact PTH, xUNL	<1	1.3 [0.9; 2.2]	1.9 [1.1; 4.4]	6.2 [2.9; 10.7]	<0.001
Total AP, U/L	35–130	63.0 [47.2; 86.8]	86.7 [69.0; 109.0]	125.0 [101.0; 182.1]	<0.001
BsAP, ug/L	6.1–25.5	15.3 [11.1; 22.1]	24.7 [16.3; 34.2]	47.4 [33.8; 66.8]	<0.001
Intact PINP, ng/mL	12.8–82.6	31.5 [23.1; 44.7]	66.1 [39.7; 95.0]	154.7 [119.0; 219.8]	<0.001
TRAP5b, U/L	1.1–6.9	2.7 [1.9; 3.4]	4.1 [2.8; 5.8]	6.4 [5.1; 8.5]	<0.001

Data are median [IQR] with p values by Kruskal Wallis equality-of-populations rank test

Abbr.: AP=alkaline phosphatase, BsAP=bone-specific alkaline phosphatase, PINP=pro-collagen type I pro-peptide, PTH=parathyroid hormone, TRAP5b=tartrate resistant acid phosphatase type 5b, xUNL=times the upper limit of normal range

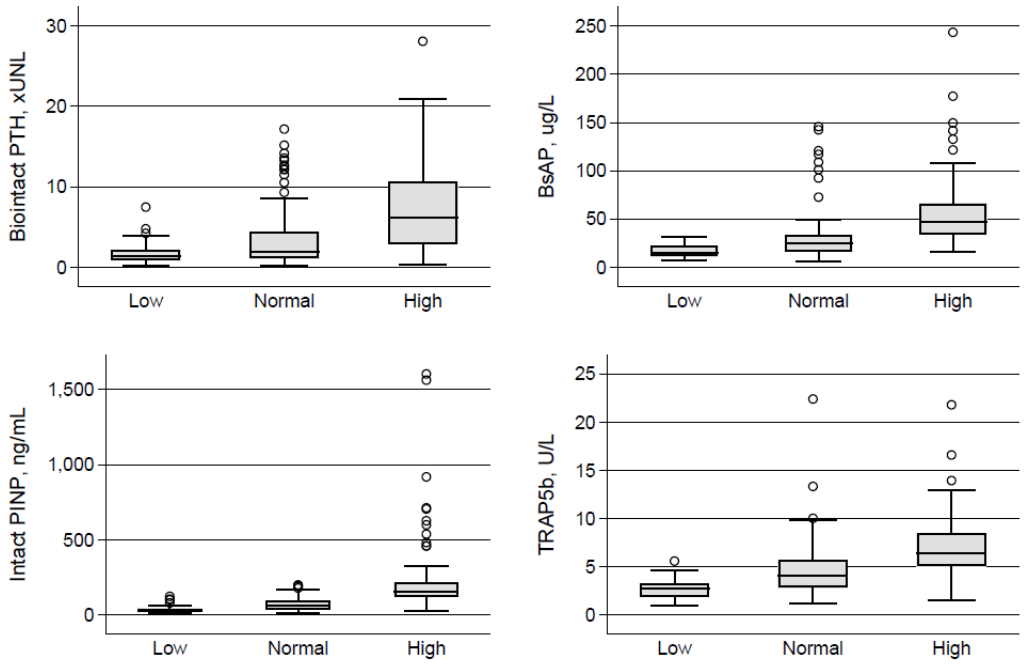

Figure 1 Distribution of biointact parathyroid hormone (PTH), bone-specific alkaline phosphatase (BsAP), procollagen type I N-terminal propeptide (PINP), and tartrate-resistant acid phosphatase isoform 5b (TRAP5b) across categories of low, normal, and hi

Moderate, highly significant correlations were seen between the levels of biochemical markers and dynamic and static parameters of remodeling (**Table** 3). The bone turnover markers were all significantly correlated to PTH (*rho* 0.45 – 0.52, $p < 0.001$), and to each other (*rho* 0.76 – 0.79, $p < 0.001$).

Table 3 Correlations between biochemical markers of bone turnover and bone histomorphometry

	Biointact PTH, ρg/mL	Total AP, U/L	BsAP, μg/L	Intact PINP, ng/mL	TRAP5b, U/L
Turnover					
Bone formation rate/Total tissue area, μm²/mm²/day	0.509***	0.520***	0.631***	0.693***	0.615***
Osteoblast/Bone perimeter, %	0.505***	0.262*	0.393***	0.434***	0.404***
Eroded/Bone perimeter, %	0.443***	0.269***	0.293***	0.366***	0.478***
Osteoclast/Bone perimeter, %	0.438***	0.260**	0.320***	0.374***	0.459***
Mineralization					
Mineralization lag time, days	-0.149	-0.201	-0.211	-0.317***	-0.305***
Osteoid/Bone area, %	0.409***	0.344***	0.450***	0.396***	0.378***
Osteoid/Bone perimeter, %	0.452***	0.305**	0.426***	0.397***	0.359***
Osteoid width, μm	0.216*	0.318***	0.342***	0.332***	0.274**

Spearman's *rho* with *p<0.05, **p<0.01, ***p<0.001 after Bonferroni adjustment for multiple comparisons

Abbr.: AP=alkaline phosphatase, BsAP=bone-specific alkaline phosphatase, PINP=procollagen type I N-terminal propeptide, PTH=parathyroid hormone, TRAP5b=tartrate resistant acid phosphatase type 5b

Diagnostic performance of biomarkers

Figure 2 shows areas under the receiver operator characteristics curve (AUCs) for separating high *vs* non-high, and low *vs* non-low bone turnover in the overall cohort. Discriminatory ability was consistently very good for both high and low bone turnover with AUCs>0.80 for the bone turnover markers while slightly lower (AUCs>0.75) for biointact PTH and total AP. Compared to biointact PTH, intact PINP was a significantly better predictor of high turnover (AUC 0.88 *vs.* 0.78, $p = 0.006$), and TRAP5b was a better predictor for low turnover (AUC 0.82 *vs.* 0.73, $p = 0.04$).

To evaluate diagnostic performance, we randomly split the cohort into separate exploration ($n = 100$) and validation ($n = 99$) subsets. The distribution of bone turnover categories, demographic characteristics, and levels of biochemical markers were balanced between the exploration and validation cohorts (**Table** S1).

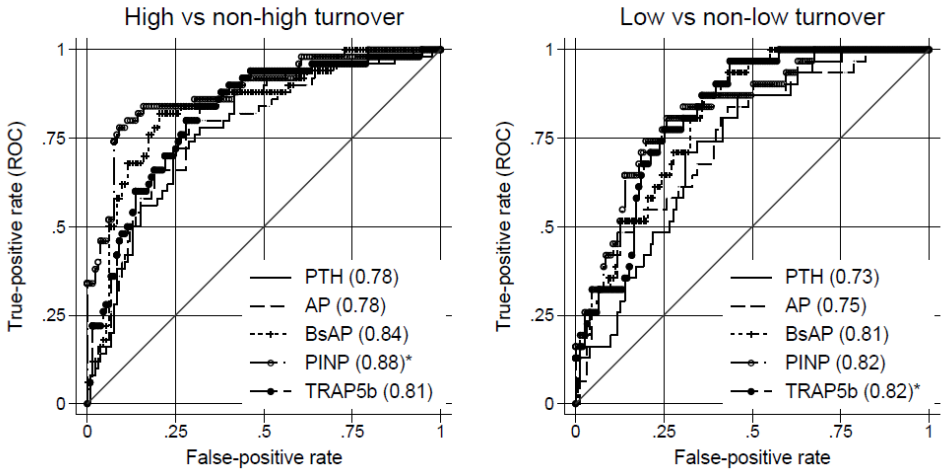

Figure 2 Areas under the receiver operator characteristics curves for the diagnosis of bone turnover by biochemical markers biointact parathyroid hormone (PTH), total and bone-specific alkaline phosphatase (tAP and BsAP), procollagen type I N-terminal pr

Optimal diagnostic cutoffs for high *vs* non-high and low *vs* non-low bone turnover were determined based on ROC curves in the exploration cohort, and then applied to the validation cohort to evaluate diagnostic performance. Results are shown in **Table** 4. Negative predictive values were high, indicating the ability to rule out both high and low bone turnover by using the suggested cutoffs. Combinations of bone turnover markers resulted in slightly higher diagnostic performance, with overall accuracies of 90% for high turnover, using the combination of Intact PINP and TRAP5b, and 78% for low turnover with the combination of BsAP and TRAP5b. Combinations of markers also resulted in high positive predictive values for a diagnosis of high turnover, indicating greater surety of the diagnosis with two biomarkers above the cutoffs.

Table 4 Diagnostic performance of biochemical markers for a diagnosis of high or low bone turnover

	Exploration cohort (*n* = 100)			Validation cohort (n = 99)			
High turnover	AUC	Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy
Biointact PTH, ρg/mL	0.78 (0.67, 0.86)	>143.5	70%	74%	57%	84%	73%
Total AP, U/L	0.77 (0.65, 0.86)	>97	76%	77%	61%	87%	77%
BsAP, ug/L	0.83 (0.73, 0.91)	>33.7	73%	86%	71%	88%	82%
Intact PINP, ng/mL	0.85 (0.74, 0.93)	>120.7	73%	94%	85%	89%	88%
TRAP5b, U/L	0.78 (0.66, 0.86)	>5.05	77%	76%	59%	88%	76%
BsAP + Intact PINP	0.84 (0.74. 0.94)	As above	63%	97%	90%	85%	86%
BsAP + TRAP5b	0.79 (0.70, 0.88)	As above	63%	91%	76%	85%	82%
PINP + TRAP5b	0.84 (0.74. 0.94)	As above	82%	94%	88%	91%	90%

Low turnover	AUC	Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy
Biointact PTH, ρg/mL	0.77 (0.65, 0.86)	<90.5	69%	52%	19%	91%	54%
Total AP, U/L	0.76 (0.63, 0.85)	<87	64%	57%	21%	90%	58%
BsAP, ug/L	0.82 (0.72, 0.90)	<24.2	87%	58%	28%	96%	63%
Intact PINP, ng/mL	0.83 (0.72, 0.91)	<49.8	80%	70%	33%	95%	72%
TRAP5b, U/L	0.84 (0.74, 0.91)	<3.44	73%	74%	34%	94%	74%
BsAP + Intact PINP	0.84 (0.75, 0.93)	As above	73%	74%	34%	94%	74%
BsAP + TRAP5b	0.86 (0.79, 0.94)	As above	60%	81%	38%	92%	78%
PINP + TRAP5b	0.85 (0.77. 0.93)	As above	67%	77%	34%	93%	72%

Area under the receiver operator characteristics curve (AUC) and cutoffs as determined by Liu's method were - calculated using the exploration cohort only. Sensitivity, specificity, negative and positive predictive values (NPV, PPV) were calculated for the validation cohort only, using the specified cutoffs. For combined biochemical markers, both markers were above/below the cutoff for a positive test result, while either above/below were counted as a negative test result

Abbr.: AP=alkaline phosphatase, BsAP=bone-specific alkaline phosphatase, PINP=pro-collagen type I pro-peptide, PTH=parathyroid hormone, TRAP5b=tartrate resistant acid phosphatase type 5b

As a sensitivity analysis, we repeated the AUC analysis separating kidney transplant candidates and recipients. Results are shown in **Table 5**. The discrimination of high bone turnover was similar comparing patients with CKD stage G4-G5D and kidney transplant recipients, while for low bone turnover, AUC values were numerically lower in patients biopsied after kidney transplantation, particularly for biointact PTH.

Table 5 Discriminatory ability of biochemical markers for high and low bone turnover stratified by kidney transplantation status

	High turnover		Low turnover	
	CKD stage G4-G5D (36 vs 44)	*Kidney transplant recipients (21 vs 98)*	*CKD stage G4-G5D (6 vs 74)*	*Kidney transplant recipients (27 vs 92)*
Biointact PTH, ρg/mL	0.66 (0.54, 0.79)	0.76 (0.63, 0.89)	0.82 (0.64, 0.99)	0.61 (0.49, 0.74)
Total AP, U/L	0.69 (0.57, 0.81)	0.74 (0.60, 0.89)	0.85 (0.73, 0.97)	0.69 (0.56, 0.81)
BsAP, ug/L	0.78 (0.67, 0.89)	0.80 (0.68, 0.92)	0.94 (0.86, 1.00)	0.74 (0.64, 0.84)
Intact PINP, ng/mL	0.84 (0.75, 0.93)	0.87 (0.77, 0.97)	0.89 (0.77, 1.00)	0.77 (0.67, 0.87)
TRAP5b, U/L	0.75 (0.64, 0.86)	0.78 (0.65, 0.91)	0.93 (0.87, 1.00)	0.74 (0.65, 0.84)

Data are area under the receiver operator characteristics curve for high vs non-high, and low vs non-low bone turnover

Abbr.: AP=alkaline phosphatase, BsAP=bone-specific alkaline phosphatase, PINP=pro-collagen type I pro-peptide, PTH=parathyroid hormone, TRAP5b=tartrate resistant acid phosphatase type 5b

Finally, to account for the effect of disease prevalence on diagnostic test interpretation, we calculated negative and positive predictive values for any possible prevalence of high and low bone turnover, i.e. from 0 to 100%. Results are shown in **Figure** S2. The ability to rule out high and low bone turnover using biomarker cutoffs was found to be acceptable (>90%) with prevalence of either condition below 30%.

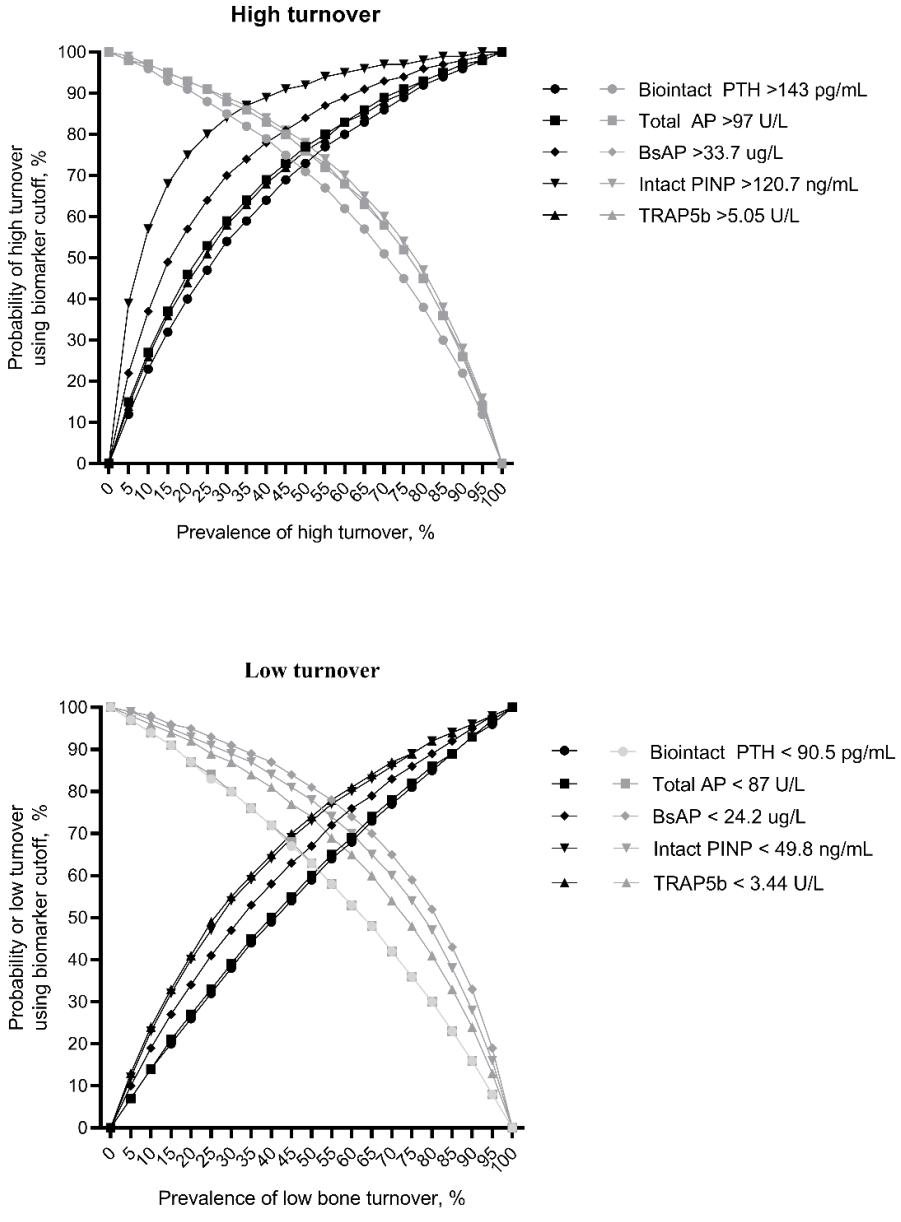

Figure S2 Negative and positive predictive values of high and low bone turnover using biomarker cutoffs, as a function of disease prevalence

Discussion

The key finding of this study is, that biochemical markers of skeletal remodeling show acceptable diagnostic accuracy for bone turnover, both in patients with CKD stages G4-G5D and in kidney transplant recipients. High negative predictive values indicate the ability to rule out the presence of high and low bone turnover, which could potentially decrease the need for an invasive bone biopsy.

The discriminatory abilities of all biochemical markers were good, with AUC values >0.80 for both high and low bone turnover using the bone turnover markers, and slightly lower values for biointact PTH and total AP. The calculated cutoffs in the exploration cohort resulted in sensitivities and specificities in the range of 70 – 95% for high turnover disease, and 60 – 85% for low turnover disease, with the highest numerical values for BsAP, Intact PINP, and TRAP5b. These results are largely in accordance with what have been published previously.[6,8,24,25]

Predictive values of negative and positive test-results are often given to illustrate clinical usefulness. High negative predictive values were seen for both high and low bone turnover, indicating the ability to rule out either condition using the suggested cutoffs. Further, with two markers above the cutoffs, positive predictive values up towards 90% were seen for high bone turnover. This could find clinical utility as an aid to treatment-decisions, for example by ruling out the presence of low turnover disease when considering initiation of anti-resorptive treatment, or estimating the likelihood of high bone turnover when considering whether to intensify treatment for secondary hyperparathyroidism. Positive predictive values for low turnover, however, were consistently very poor and did not improve by combining markers.

While BsAP has been extensively investigated,[6–8,26–29] only a few studies previously examined the diagnostic value of the more novel markers Intact PINP and TRAP5b.[24,25] Sprague *et al*[6] included PINP in their multicenter study of diagnostic accuracy of biomarkers in patients with kidney failure, but did not measure the intact, trimeric form. As monomers of PINP accumulate in CKD, the total PINP analysis is affected by kidney function;[30] in the study by Sprague *et al*, any residual kidney function in the participating dialysis patients could have affected the results. Salam *et al*[24] reported the diagnostic performance of a panel of bone

turnover markers in patients with CKD stages G4-G5D. Discrimination of low turnover was found to be good, particularly for BsAP, Intact PINP, and TRAP5b (AUCs 0.79 – 0.80) while significantly less so for intact PTH (AUC 0.56). We note that the calculated cutoffs for high and low turnover reported by Salam *et al.*[24] were very similar to what we demonstrate in the present study, which shows consistency across different study populations.

We hypothesized that the bone turnover markers would provide a better diagnostic performance when compared to PTH. As BsAP, PINP and TRAP5b are released from the bone tissue during the skeletal remodeling process, they have the potential to deliver highly specific information on whole-skeletal bone turnover. In contrast, PTH is a main regulator of skeletal remodeling, and may be competing with other effectors on the bone, such as hypogonadism, steroid exposure, inflammation, and uremic toxins,[31] leading to a poorer prediction of the current skeletal remodeling rate. However, while there were indications of better diagnostic accuracy for the bone turnover markers, with higher AUC values, the differences in diagnostic performance as judged by the values of negative and positive test results, were negligible. Similarly, Salam *et al.*[24] found significantly better prediction of low bone turnover by bone turnover markers as compared to intact PTH in patients with CKD stage G4-G5D, but again, the effect on test performance was small. The intact PTH assay capture not only the full-length 1-84 PTH, but also various PTH fragments, and as these are retained in CKD,[32,33] PTH measured by the intact assay could be expected to perform less well, particularly in cohorts spanning different CKD stages. As we measured biointact 1-84 PTH in this study, any accumulation of fragments should not affect our results. It remains uncertain whether there is a benefit of measuring the complete hormone; in the before-mentioned study by Sprague et al.[6], no difference in diagnostic accuracy for bone turnover was found for biointact *vs.* intact PTH.

Diagnostic performance of biomarkers for high bone turnover was comparable for patients in CKD stage G4 to G5D and kidney transplant recipients – but with numerically lower AUCs for low bone turnover in post-transplant patients. The post-transplant bone phenotype is a composite of renal osteodystrophy pre-transplant, resolving or ongoing disturbances of mineral metabolism, and effects of immunosuppressants on bone. Profound alterations in mineral metabolism occur after kidney transplantation, and although kidney

function and biochemical parameters generally stabilize by 3 months,[34,35] persistent disturbances are seen in subsets of patients throughout the first post-transplant year.[36,37] Marked variability in the histomorphometric pattern, even within the same bone sample, has been demonstrated in kidney transplant recipients,[38] and may reflect a slow transition to a new skeletal steady state. In contrast, biochemical markers of bone turnover are highly dynamic, as seen by the rapid changes in circulating levels in response to fracture[39] and anti-osteoporotic treatment[40]. This time lag between bone turnover as evaluated by histomorphometry *vs* biochemistry may contribute to a lower diagnostic accuracy of biomarkers in the post-transplant setting. A steroid-minimization protocol was utilized in the study period. Still, 74% of patients received oral prednisolone at the time of the bone biopsy. Glucocorticoids result in a sustained depression of osteoblast function, with more transient increase in osteoclast activity.[41] Thus, the effect of glucocorticoids may contribute to a more severe phenotype of low bone turnover post-transplant.

The main strength of this study is the considerable number of bone biopsies available for evaluation. Further, we limited our analyses to high quality, successfully labelled bone biopsies, and all histomorphometric analyses were performed at the same laboratory, avoiding variability in sample processing, reference cutoffs, and diagnostic practice. To reduce optimism bias when evaluating diagnostic performance, we used an explorative subset of patients for estimating the diagnostic cutoffs, which were then validated in a separate subset. However, no external validation was performed which, together with the single-center approach, and ethnically homogenous cohort, may reduce the reproducibility of our findings. The heterogeneity in our cohort may be considered a limitation, as we included patients in CKD stage G4-G5, patients receiving dialysis therapy, as well as kidney transplant recipients. However, we are not aware that the stage of CKD should affect the relationship between skeletal remodeling and release of biomarkers from the bone tissue, and as already detailed, the biomarkers included in this study does not accumulate with decreasing kidney function.[13] Blood sampling in patients with CKD stage G5D was random with regards to the last dialysis session, but the effect of hemodialysis therapy on circulating levels of bone turnover markers has previously been shown to be limited.[42,43] Any effect of peritoneal dialysis treatment on circulating biomarker levels has,

to our knowledge, not yet been investigated; but considering the continuous nature of this treatment, it should likewise be minimal.

An inherent limitation to these analyses is the consideration that the circulating bone turnover markers must represent whole skeletal metabolism, including both the central and distal skeleton and cortical and trabecular bone compartments. In contrast, the bone biopsy is representative of a single skeletal site only and the trabecular bone compartment specifically. In accordance, rather weak correlations were found between bone biomarkers and histomorphometric parameters in post-menopausal women.[44] Recent pilot trials highlight the potential of bone turnover markers to deliver dynamic information, with changes in circulating levels reflecting the treatment effects on skeletal remodeling[45,46]; a clear advantage as opposed to the bone biopsy, which cannot easily be repeated. Lastly, the predictive ability of these markers on relevant clinical outcomes is yet to be established and will likely require the collaborative effort of multiple centers with expertize within this field.[47]

Supplementary material

Table S1: Balance of demographic and biochemical variables in the validation and exploration cohorts

Figure S1 Flowchart of patient inclusion and exclusion

Figure S2: Negative and positive predictive values of high and low bone turnover using biomarker cutoffs, as a function of prevalence of disease

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Authors' Contributions

Research idea and study design: PE, EC, PDH; data acquisition: GB, LV, BB, KC, BM, MN, BS, DK; data analysis/interpretation: HSJ, GB, PDH, EC, PE; statistical analysis: HSJ; supervision or mentorship: PE. Each author contributed important intellectual content during manuscript drafting or revision and agrees to be personally accountable for the individual's own contributions and to ensure that questions pertaining to the accuracy or integrity of any portion of the work, even one in which the author was not directly involved, are appropriately investigated and resolved, including with documentation in the literature if appropriate.

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Supplementary material

Table S1 Demographic and biochemical variables in exploration and validation cohorts

	Exploration cohort (n = 100)	Validation cohort (n = 99)	P-value
Age, years	56 ± 12	55 ± 13	0.75
Gender, male	63 (63%)	71 (72%)	0.19
Diabetes mellitus, any type	21 (21%)	24 (24%)	0.58
Previous parathyroidectomy	13 (13%)	8 (8%)	0.27
Body mass index, kg/m²	25.7 ± 4.9	24.2 ± 4.4	0.09
Stage of CKD			
CKD stage G4-G5D	37 (37%)	43 (43%)	
Kidney transplant recipient	63 (63%)	56 (57%)	0.35
Bone turnover			
Low	18 (18%)	15 (15%)	
Normal	58 (58%)	51 (52%)	
High	24 (24%)	33 (33%)	0.34
Biointact parathyroid hormone, ρg/mL	98.7 [49.7; 225.8]	87.3 [44.0; 234.0]	0.83
Total alkaline phosphatase, U/L	92.0 [72.7; 124.3]	88.2 [66.2; 119.0]	0.43
Total calcium, mg/dL	9.71 ± 0.76	9.72 ± 0.85	0.93
Phosphate, mg/dL	4.02 ± 1.73	4.08 ± 1.79	0.83
Calciciol, ng/mL	34 ± 13	38 ± 16	0.09
BsAP, ug/L	26.6 [17.1; 40.1]	24.8 [15.8; 39.7]	0.33
Intact PINP, ng/mL	75.2 [40.3; 137.3]	70.1 [37.8; 133.8]	0.77
TRAP5b, U/L	4.6 [2.8; 6.4]	4.4 [2.8; 6.3]	0.91
Mimpara use	3 (3%)	5 (5%)	0.46
Vitamin D use	55 (55%)	53 (54%)	0.84
Active vitamin D use	31 (31%)	31 (31%)	0.96
Methylprednisolone use	53 (53%)	53 (54%)	0.94

Data are mean ± SD, median [IQR], or n (%), with p values by Student's t test, Wilcoxon ranksum test, or Pearson's X^2 test, respectively

Figure S1 Flow of patient inclusion and exclusion