Non-oxidized parathyroid hormone (PTH) measured by current method is not superior to total PTH in assessing bone turnover in chronic kidney disease

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Parathyroid hormone (PTH) is a key regulator of bone turnover but can be oxidized *in vivo*, which impairs biological activity. Variable PTH oxidation may account for the rather poor correlation of PTH with indices of bone turnover in chronic kidney disease. Here, we tested whether non-oxidized PTH is superior to total PTH as a marker of bone turnover in 31 patients with kidney failure included from an ongoing prospective observational bone biopsy study and selected to cover the whole spectrum of bone turnover. Receiver Operating Characteristic (ROC) curves, Spearman correlation and regression analysis of non-oxidized PTH, total PTH and bone turnover markers (bone-specific alkaline phosphatase, procollagen N-terminal pro-peptide and tartrate-resistant acid phosphatase 5b) were used to assess the capability of non-oxidized PTH vs. total PTH to discriminate low from non-low and high from non-high bone turnover, as assessed quantitatively by bone histomorphometry. Serum levels of non-oxidized PTH and total PTH were strongly and significantly correlated. Histomorphometric parameters of bone turnover and the circulating bone turnover markers showed similar correlation coefficients with non-oxidized PTH and total PTH. The area under the ROC (AUROC) values for discriminating between low/non-low turnover for non-oxidized PTH and total PTH were significant and comparable (0.82 and 0.79, respectively). For high/non-high turnover the AUROCs were also significant and of the same magnitude (0.76 and 0.80, respectively). Thus, measuring non-oxidized PTH using the currently available method provides no added value compared to total PTH as an indicator of bone turnover in patients with kidney failure.

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Received 22 October 2020; revised 2 December 2020; accepted 10 December 2020; published online 8 January 2021

Kidney International (2021) **99,** 1173–1178; https://doi.org/10.1016/j.kint.2020.12.024

KEYWORDS: bone histomorphometry; bone turnover; chronic kidney disease; non-oxidized PTH; parathyroid hormone

Parathyroid hormone (PTH) is a key diagnostic and prognostic marker in patients with chronic kidney disease (CKD) and mineral and bone disorders.[1] However, the correlation of PTH with underlying bone turnover, as well as mortality and morbidity, is weak, especially in the middle range of Kidney Disease: Improving Global Outcomes targets.[1] This weak correlation may partially be explained by the variable post-translational oxidation of the hormone.[2,3]

PTH is a peptide hormone, consisting of 84 amino acids. The amino acids on the 8th and 18th position are methionine residues. Methionine is prone to oxidation, resulting in methionine sulfoxide. The methionine residues on PTH can be oxidized *in vivo*.[4] This oxidation results in conformational changes, reducing the biological potency of PTH to activate the PTH-1 receptor.[5] As recently reviewed, *in vivo* and *in vitro* studies in a wide variety of animals have shown that the oxidation of both methionine residues diminishes the biological function of PTH.[5] However, contemporary PTH assays have been developed with an emphasis on measuring full-length PTH instead of PTH fragments but do not discriminate between non-oxidized PTH (n-oxPTH) and oxidized PTH (oxPTH).

A method to separate n-oxPTH from total PTH (tPTH) and assess n-oxPTH concentrations quantitatively was first described in 2012.[2] First, oxPTH is selectively extracted by specific antibodies in an affinity column. Subsequently, n-oxPTH is measured with routine (automated) PTH immunoassays in the remaining sample. With the introduction of the n-oxPTH measurement, intriguing questions have arisen

about its added value. Although experimental data clearly reveal reduced biological activity of oxPTH, there is no consensus as to whether measuring n-oxPTH confers diagnostic superiority.

Two previous studies—1 in patients with CKD stage 5 and 1 in patients with CKD stage 2 to 4—have shown that tPTH was more strongly associated with mortality than n-oxPTH.[6,7] It was concluded that oxidative stress, of which tPTH was assumed to be a reflection, is probably a more powerful predictor of mortality.[6,8] As the potential clinical added value of n-oxPTH therefore would not reside in predicting mortality and morbidity, it is hypothesized that n-oxPTH may be superior to tPTH in predicting bone turnover.

The purpose of this study was to explore whether n-oxPTH outperforms tPTH as a biomarker of bone turnover in end-stage kidney disease (ESKD).

METHODS

Patients and study protocol

Adult Caucasian patients (age > 18 years) with ESKD were recruited from an ongoing, prospective, observational study on the evolution of bone histomorphometry before renal transplantation (ClinicalTrials.gov identifier: NCT01886950) at the University Hospitals Leuven. For the present analysis, we selected 10 patients with ESKD and low bone turnover, 10 patients with ESKD and normal bone turnover, and 11 patients with ESKD and high bone turnover according to histomorphometric criteria (see below). None of the patients had a history of therapy with antiresorptive drugs, and 3 patients had a history of parathyroidectomy (time elapsed between the procedure and bone biopsy was 7, 23, and 82 months, respectively). The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospitals Leuven, Belgium. Informed consent was obtained from all patients.

Biochemical analysis

Blood samples were collected (random, nonfasted) at the time of bone biopsy. After centrifugation, serum was stored at −80 °C until further analysis.

tPTH was measured in ethylenediamine tetraacetic acid (EDTA) plasma using an automated second-generation PTH immunoassay (Cobas, Roche Diagnostics, Rotkreuz, Switzerland), with an intra- and interassay coefficient of variation of <2.7% and <6.5%, respectively. n-oxPTH concentrations were measured using an oxPTH affinity column (A1112, Immundiagnostik AG, Bensheim, Germany) as described previously.[4] In short, oxPTH is removed by affinity chromatography columns filled with beads coated with antibodies against all oxidized forms of PTH. The columns were filled with 300 µl of plasma and incubated end-over-end at room temperature for 1 hour. Afterward, we determined n-oxPTH in the eluate by using the same Cobas PTH immunoassay as described above. The interassay coefficient of variation of the n-oxPTH measurement at concentrations of <2 and >2 pmol/l is 10% and 2.4%, respectively.

25-Hydroxy vitamin D (calcidiol) concentrations were measured using in-house developed radioimmunoassays as described previously.[9] Bone-specific alkaline phosphatase, tartrate-resistant acid phosphatase 5b, and intact N-terminal propeptide of type I procollagen were measured using the IDS-iSYS instrument (IDS, Boldon, UK). Inter- and intra-assay coefficient of variations were <10% for all assays.

Table 1 | Baseline characteristics (*N* = 31)

Parameter	Value
Age (yr)	52 ± 14
Sex: male	20 (65)
Systolic blood pressure (mm Hg)	143 ± 23
Diastolic blood pressure (mm Hg)	85 ± 20
Type 1 diabetes mellitus	1 (3)
Type 2 diabetes mellitus	1 (3)
Parathyroidectomy	3 (10)
HD/PD	18 (58)/13 (42)
Dialysis vintage (mo)	18 (8–41)
Low bone turnover	10 (32)
Normal bone turnover	10 (32)
High bone turnover	11 (35)
Serum parameters	
tPTH (pmol/l)	26.6 (16.1–66.5)
n-oxPTH (pmol/l)	6.7 (2.3–8.6)
Bone ALP (µg/l)	33.3 (16.2–60.8)
PINP (µg/l)	76 (39–165)
TRACP5b (U/l)	5.8 ± 3.0
Calcium (mmol/l) (*n* = 26)	2.40 (2.28–2.47)
Phosphate (mmol/l) (*n* = 26)	1.75 (1.39–2.13)
Structural histomorphometric parameters	
Bone area (%)	17.9 (16.3–25.3)
Trabecular thickness (µm)	137 (117–152)
Trabecular number (mm^{-1})	1.85 (1.48–2.17)
Trabecular spacing (µm)	405 (293–545)
Static histomorphometric remodeling parameters	
Osteoid area (%)	2.6 (0.97–5.1)
Osteoid perimeter (%)	20.5 (10.8–35.2)
Osteoid width (µm)	8.7 (7.0–10.9)
Eroded perimeter (%)	5.0 (2.1–9.0)
Osteoblast perimeter/total perimeter (%)	3.0 (0.34–8.6)
Osteoclast perimeter/total perimeter (%)	1.0 (0.05–2.0)
Dynamic histomorphometric remodeling parameters	
Mineralized bone area (%)	17 (16–24)
Mineral apposition rate (µm/d) (*n* = 25)	1.02 (0.78–1.24)
Bone formation rate ($\mu m^2/mm^2$/d) (*n* = 25)	342 (135–899)
Adjusted apposition rate (µm/d) (*n* = 25)	0.56 (0.36–1.39)
Osteoid maturation time (d) (*n* = 25)	9.7 (6.4–11.2)

ALP, alkaline phosphatase; HD, hemodialysis; PD, peritoneal dialysis; n-oxPTH, non-oxidized parathyroid hormone; PINP, total procollagen type 1 N-terminal propeptide; tPTH, total parathyroid hormone; TRACP5b, tartrate-resistant acid phosphatase 5b. Data are expressed as mean ± SD, median (interquartile range), or n (%).

Bone histomorphometry

Transiliac bone biopsies were performed using a trephine needle with an internal diameter of 4.5 mm (Osteobell, Mirandola, Italy, and Biopsybell, Mirandola, Italy).[10] All but 6 patients underwent two 3-day oral tetracycline (2 × 500 mg/d) administration sessions, with in-between an 11-day tetracycline-free interval before the bone biopsy procedure.

Bone biopsies were fixed in 70% ethanol and embedded in a methyl metacrylate resin. For light microscopic examination of static bone parameters, the modified Goldner technique was used to stain the nondecalcified 5-µm-thick sections. For fluorescence microscopic examination of dynamic bone parameters, 10-µm-thick sections were mounted unstained in 100% glycerol to visualize the tetracycline labels. The results are reported as 2-dimensional

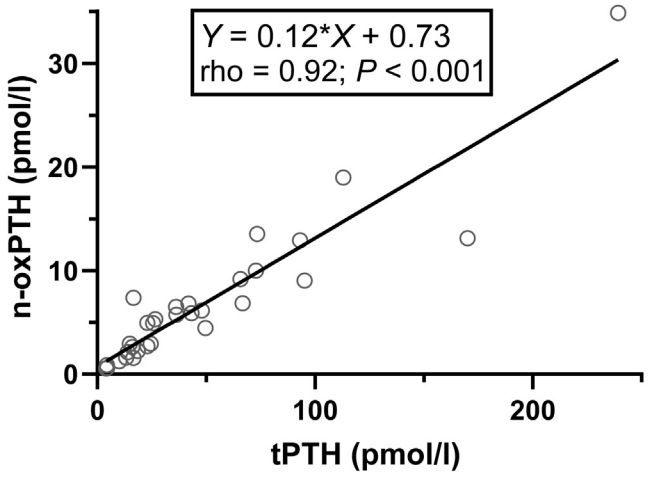

Figure 1 | Univariate linear regressions for non-oxidized parathyroid hormone (n-oxPTH) and total parathyroid hormone (tPTH) ($N = 31$).

measurements using nomenclature established by the American Society for Bone and Mineral Research.[11] The following criteria were used to define the turnover category: high turnover, bone formation rate (BFR) > 613 $\mu m^2/mm^2/d$; low turnover, BFR < 97 $\mu m^2/mm^2/d$ and/or absence of cellular activity (osteoblast perimeter/total perimeter and osteoclast perimeter/total perimeter <1%). Patients not belonging to either group were classified as "those with normal bone turnover."

Statistical analysis

Data following a normal distribution are expressed as mean ± SD, and data following a skewed distribution are expressed as median with interquartile range (IQR). Skewed variables were normalized before parametric analyses.

Spearman correlation coefficients and univariate linear regressions were used to evaluate the relationship between n-oxPTH, tPTH, bone histomorphometric parameters, and biochemical parameters. The receiver operating characteristic curve and area under the receiver operating characteristic (AUROC) curve were used to assess the diagnostic performance of n-oxPTH versus tPTH to discriminate low from non-low and high from non-high bone turnover.

P values < 0.05 were considered to reflect statistical significance. Statistical analysis was performed using MedCalc Statistical Software version 18.5 (MedCalc Software bvba, Ostend, Belgium).

RESULTS

Baseline characteristics

Demographic and clinical characteristics of the included participants ($N = 31$) are summarized in Table 1. The baseline characteristics stratified by bone turnover group are summarized in Supplementary Table S1. The mean age was 52 ± 14 years, and 65% of the participants were men. One patient had a history of type 1 diabetes mellitus, 1 had a history of type 2 diabetes mellitus, and 3 had previously undergone parathyroidectomy. The median dialysis vintage was 18 months (IQR 8–41 months). The median bone formation rate was 342 $\mu m^2/mm^2/d$ (IQR 135–899 $\mu m^2/mm^2/d$). The median n-oxPTH and tPTH concentrations were 2.3 pmol/l (IQR 1.1–5.4 pmol/l) and 18.3 pmol/l (IQR 9.1–54.0 pmol/l), respectively. Although there were relatively more patients undergoing hemodialysis than peritoneal dialysis in the high bone turnover group, their serum levels of n-oxPTH and tPTH were of comparable magnitude.

Table 2 | Spearman correlations of n-oxPTH vs. tPTH in ESKD ($N = 31$)

	n-oxPTH		tPTH	
Parameter	**ρ**	**P**	**ρ**	**P**
Serum parameters				
tPTH	0.92	<0.001		
Bone ALP	0.61	<0.001	0.71	<0.001
PINP	0.69	<0.001	0.68	<0.001
TRACP5b	0.64	<0.001	0.65	<0.001
Structural histomorphometric parameters				
Bone area	0.03	0.85	0.02	0.91
Trabecular thickness	0.31	0.09	0.39	0.03
Trabecular number	−0.09	0.65	−0.15	0.43
Trabecular spacing	0.08	0.66	0.14	0.45
Static histomorphometric remodeling parameters				
Osteoid area	0.28	0.13	0.35	0.06
Osteoid perimeter	0.30	0.10	0.36	0.04
Osteoid width	0.31	0.09	0.43	0.02
Eroded perimeter	0.64	<0.001	0.62	<0.001
Osteoblast perimeter/total perimeter	0.55	0.001	0.53	0.002
Osteoclast perimeter/total perimeter	0.58	<0.001	0.58	<0.001
Dynamic histomorphometric remodeling parameters				
Mineralized bone area	0.02	0.92	-0.01	0.97
Mineral apposition rate ($n = 25$)	0.66	<0.001	0.58	<0.001
Bone formation rate ($n = 25$)	0.54	0.006	0.61	0.001
Adjusted apposition rate ($n = 25$)	0.38	0.06	0.43	0.03
Osteoid maturation time ($n = 25$)	−0.09	0.67	0.09	0.69

ALP, alkaline phosphatase; ESKD, end-stage kidney disease; n-oxPTH, non-oxidized parathyroid hormone; PINP, total procollagen type 1 N-terminal propeptide; tPTH, total parathyroid hormone; TRACP5b, tartrate-resistant acid phosphatase 5b.

Correlation analysis

The Spearman correlation coefficient between n-oxPTH and tPTH was 0.92 ($P < 0.001$). As Figure 1 shows, the regression analysis equation was linear with the formula n-oxPTH = 0.12 × tPTH + 0.73. Hence, on average, n-oxPTH values were 12% (95% confidence interval 11%–14%) of tPTH.

Table 2 presents the correlations of n-oxPTH and tPTH with serum bone turnover markers, structural histomorphometric parameters, and static and dynamic histomorphometric remodeling parameters. Serum bone formation markers, that is, bone-specific alkaline phosphatase and total procollagen type 1 N-terminal propeptide, all showed significant correlations with both n-oxPTH and tPTH, with correlation coefficients ranging between 0.61 and 0.71. Structural histomorphometric parameters did not exhibit a significant correlation with n-oxPTH. One of the most frequently used dynamic histomorphometric bone remodeling parameters—the bone formation rate—showed a correlation with n-oxPTH of 0.54 ($P = 0.006$) and with tPTH of 0.61 ($P < 0.001$). Overall, correlations were numerically stronger for tPTH than for n-oxPTH, except for the mineral

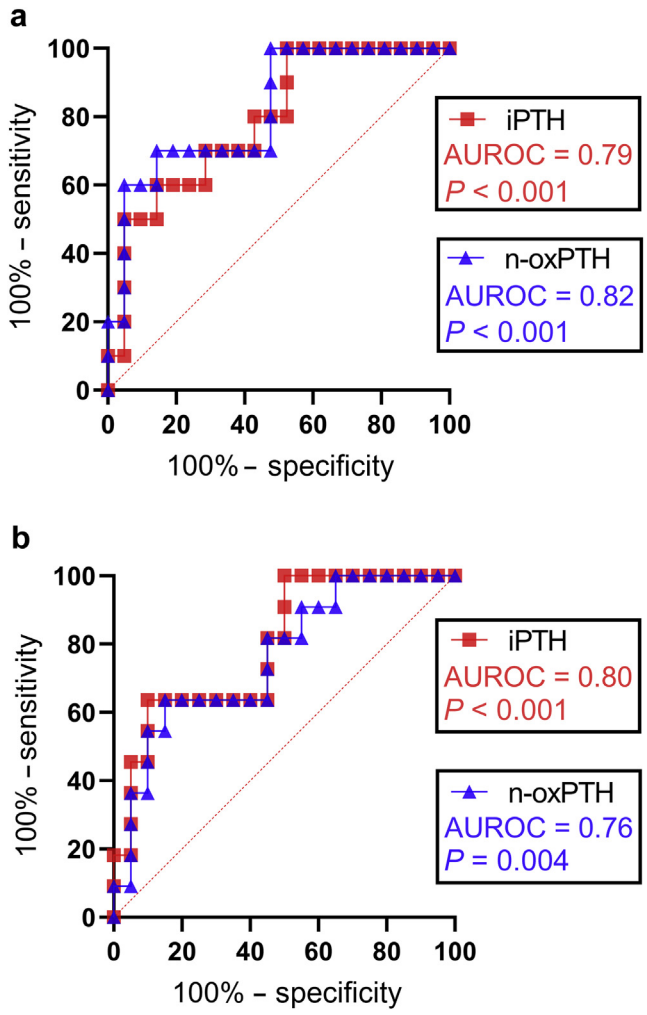

Figure 2 | Receiver operating characteristics curve for non-oxidized parathyroid hormone (n-oxPTH) and total parathyroid hormone (tPTH) (a) discriminating between low and non-low bone turnover and (b) discriminating between high and non-high bone turnover. AUROC, area under the receiver operating characteristic curve; iPTH, intact parathyroid hormone.

apposition rate (n-oxPTH: $\rho = 0.66$, $P < 0.001$; tPTH: $\rho = 0.58$, $P < 0.001$).

The diagnostic ability of n-oxPTH versus tPTH in discriminating between high and low bone turnover was assessed by receiver operating characteristic curves, as depicted in Figure 2 and Table 3. The AUROC for discriminating low versus non-low turnover was 0.82 ($P < 0.001$) for n-oxPTH and 0.79 ($P = 0.001$) for tPTH. These AUROCs showed no statistically significant difference ($P = 0.32$). The high versus non-high turnover AUROC was 0.76 ($P = 0.004$) for n-oxPTH and 0.80 ($P < 0.001$) for tPTH. These AUROCs showed no statistically significant difference either ($P = 0.32$). The AUROCs for bone-specific alkaline phosphatase, total procollagen type 1 N-terminal propeptide, and tartrate-resistant acid phosphatase 5b are presented in Table 3. The parameter with the highest AUROC for differentiating low from non-low turnover was total procollagen type 1 N-terminal propeptide (AUROC 0.862). For high versus non-high turnover, the AUROC was highest for bone-specific alkaline phosphatase (AUROC 0.909).

Table 3 | AUROC for bone turnover in ESKD ($N = 31$)

Parameter	AUROC	SE	95% CI
Low ($n = 10$) vs. non-low ($n = 21$) turnover			
n-oxPTH	0.824	0.082	0.645–0.935
tPTH	0.790	0.086	0.607–0.915
BAP	0.833	0.073	0.656–0.942
PINP	0.862	0.086	0.691–0.959
TRACP5b	0.852	0.075	0.679–0.953
High ($n = 11$) vs non-high ($n = 20$) turnover			
n-oxPTH	0.764	0.091	0.577–0.897
tPTH	0.795	0.085	0.613–0.918
BAP	0.909	0.052	0.750–0.982
PINP	0.864	0.064	0.693–0.960
TRACP5b	0.882	0.062	0.715–0.969

AUROC, area under the receiver operating characteristic curve; BAP, bone-specific alkaline phosphatase; CI, confidence interval; ESKD, end-stage kidney disease; n-oxPTH, non-oxidized parathyroid hormone; PINP, total procollagen type 1 N-terminal propeptide; tPTH, total parathyroid hormone; TRACP5b, tartrate-resistant acid phosphatase 5b.

DISCUSSION

The key finding of the present study is that n-oxPTH is not superior to tPTH as a biomarker of bone turnover, defined by its criterion standard, in patients with ESKD.

Fracture risk is excessively high in patients with advanced CKD.[12] Knowledge of bone turnover is of utmost importance in defining the optimal treatment strategy in patients with advanced CKD and osteoporosis. The criterion standard for quantifying bone turnover is the histomorphometric analysis of a tetracycline double-labeled bone biopsy. Taking a bone biopsy, however, is an invasive procedure, and histopathological expertise is laborious and not widely available. As such, quantifying bone turnover by bone histomorphometry is not feasible in many routine clinical practices, and simpler methods to assess bone turnover represent an unmet clinical need.[13]

PTH is a key regulator of bone metabolism, and for decades, monitoring PTH values has been the *lingua franca* of renal bone disease management. The wide availability of rapid and automated PTH immunoassays contributed to the success of PTH as a biomarker. Data from a cross-sectional retrospective diagnostic test study, including >450 patients with bone histomorphometry data, showed an AUROC between PTH and BFR/bone surface ranging between 0.70 and 0.80, indicating only a moderate diagnostic accuracy.[14] This was also demonstrated by another cross-sectional study of 43 patients with CKD, in which the AUROC between PTH and BFR/bone surface was 0.76, indicating high turnover, but only 0.61, indicating low turnover.[15]

The oxidation of PTH results in partial or complete loss of its biological activity.[5] Conventional PTH assays cannot distinguish between oxidized and n-oxPTH. This background

raised the hypothesis to which extent measuring n-oxPTH confers superior diagnostic accuracy in discriminating between bone turnover categories, also as the majority of research concerning n-oxPTH measurements has been performed by a single research group.

Our data clearly refute this hypothesis. tPTH and n-oxPTH showed a high and almost identical AUROC, fluctuating ~0.8 for discriminating low from non-low and high from non-high bone turnover. Both tPTH and n-oxPTH showed strong correlations with histomorphometric and biochemical parameters of bone turnover. These findings were not surprising, acknowledging the very high correlation between n-oxPTH and tPTH ($\rho = 0.92$; $P < 0.001$). This strong correlation seems to be specific to the hemodialysis setting.[4,6,16] Weaker correlations have indeed been reported in healthy controls and in patients with CKD stage 2 to 4.[4,7,17] This discrepancy might be related to differences in redox status and metabolic clearance of oxPTH and n-oxPTH across stages of CKD.[18–20]

Theoretically, the ratio of PTH that is not oxidized could be calculated as the percentage of n-oxPTH/tPTH. However, as the affinity of the antibodies used in the tPTH assays may differ for different PTH species on the basis of oxidation status, this highly influences the results and the n-oxPTH/tPTH values, as well as oxPTH values, cannot be calculated.[4] This hinders studies investigating determinants of the oxidizing rate of PTH. In addition, as there is no standard of n-oxPTH or oxPTH, yet n-oxPTH or tPTH concentrations combined with a calculated ratio can only be used to assess longitudinal differences over time using the same PTH immunoassay.[5]

The results of this study should be interpreted in light of its strengths and limitations. A major strength of the present study is the availability of bone biopsy data, the criterion standard for assessing bone turnover, covering the spectrum of bone turnover from low to high. Moreover, bone turnover is assessed by a panel of bone formation and resorption biomarkers. However, the sample size, though substantial for a bone biopsy study, is rather small. The high correlation between n-oxPTH and tPTH renders a type II statistical error highly unlikely. Hence, we are convinced that extending the study population would not affect conclusions. Caution, however, is warranted when extrapolating the results to patients without ESKD and to patients with a different ethnic background, as this study included only Caucasians.

The antibodies coated on the beads of the affinity column to capture oxPTH are raised against all forms of oxPTH, that is, PTH oxidized at the 18th, 8th, or both methionine residues. Previous studies have shown that the most easily oxidized methionine residue is methionine residue 18 because of its location. Methionine residue 8 is more difficult to become oxidized as it is positioned in a hydrophobic pocket.[21] The amount of oxPTH oxidized in serum at either 1 or both residues is currently unknown. What is known is that if these forms are separated and tested for their activity, PTH oxidized solely at methionine residue 18 has residual biological activity.[5,22] This was also recently corroborated by a study assessing the FGF23 stimulating effects of several oxPTH forms.[23] As we do not know the amount of PTH oxidized at methionine residue 18, compared with PTH oxidized at methionine residue 8 or PTH oxidized at methionine residues 8 and 18, the quantification of biologically active PTH by the methods used in the present study could be an underestimation. One could speculate that separation of the fragments may result in more optimal interpretation of PTH results. However, this remains to be investigated as soon as methods are available for specific measurement, such as a liquid chromatography with tandem mass spectrometry method.

In conclusion, our data demonstrate that in patients with ESKD measuring n-oxPTH was not superior compared to tPTH in discriminating high from non-high and low from non-low turnover. In addition, other histomorphometric and serum parameters of bone turnover showed no stronger correlation to n-oxPTH than to tPTH. Therefore, using the currently available method, there is at present no added value of measuring n-oxPTH in ESKD.

DISCLOSURE

PE is on scientific advisory boards of Amgen, Vifor Fresenius Medical Care, and MEDICE Verwaltungs-GmbH. PCDH is on a scientific advisory board of Vifor Pharma and received research grants from Vifor Pharma, Inositec, OxThera, Rockwell Medical, and Shire (Takeda). MGV is on advisory boards of Amgen, Vifor Fresenius Medical Care, Kyowa Kirin, and Medice and received research support from Amgen, FMC, and Vifor-OPKO. EC is a consultant for DiaSorin, IDS, Fujirebio, bioMérieux, IDS, Menarini, and Nittobo. All the other authors declared no competing interests.

ACKNOWLEDGMENTS

Immundiagnostik AG (Bensheim, Germany) provided the oxidized parathyroid hormone affinity columns (A1112).

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Table S1. Baseline characteristics stratified per bone turnover classification.

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