

RESEARCH

The effect of vitamin D supplementation on plasma non-oxidised PTH in a randomised clinical trial

Stan Ursem^{1,*}, Vito Francic^{2,*}, Martin Keppel³, Verena Schwetz², Christian Trummer², Marlene Pandis², Felix Aberer², Martin R Gröbler², Nicolas D Verheyen⁴, Winfried März⁵, Andreas Tomaschitz⁶, Stefan Pilz², Barbara Obermayer-Pietsch² and Annemieke C Heijboer^{1,7}

¹Department of Clinical Chemistry, Amsterdam Gastroenterology & Metabolism, Amsterdam UMC, Vrije Universiteit Amsterdam, Endocrine Laboratory, Amsterdam, Netherlands

²Division of Endocrinology and Diabetology, Department of Internal Medicine, Endocrinology Lab Platform, Medical University of Graz, Graz, Austria

³University Institute for Medical and Chemical Laboratory Diagnostics, Paracelsus Medical University, Salzburg, Austria

⁴Division of Cardiology, Department of Internal Medicine, Medical University of Graz, Graz, Austria

⁵Synlab Academy, Synlab Holding Germany GmbH, München, Germany

⁶Specialist Clinic of Rehabilitation Bad Gleichenberg, Bad Gleichenberg, Austria

⁷Department of Clinical Chemistry, Amsterdam Gastroenterology & Metabolism, Amsterdam UMC, University of Amsterdam, Endocrine Laboratory, Amsterdam, Netherlands

Correspondence should be addressed to A C Heijboer: a.heijboer@vumc.nl

*(S Ursem and V Francic contributed equally to this work)

Abstract

Objective: PTH can be oxidised *in vivo*, rendering it biologically inactive. Non-oxidised PTH (n-oxPTH) may therefore give a better image of the hormonal status of the patient. While vitamin D supplementation decreases total PTH (tPTH) concentration, the effect on n-oxPTH concentration is unexplored. We investigated the effect of vitamin D on n-oxPTH concentration in comparison to tPTH and compared the correlations between parameters of calcium, bone and lipid metabolism with n-oxPTH and tPTH.

Methods: N-oxPTH was measured in 108 vitamin D-insufficient (25(OH)D <75 nmol/L) hypertensive patients, treated with vitamin D (2800 IE daily) or placebo for 8 weeks in the Styrian Vitamin D Hypertension Trial (NCT02136771). We calculated the treatment effect and performed correlation analyses of n-oxPTH and tPTH with parameters of calcium, bone and lipid metabolism and oxidative stress.

Results: After treatment, compared to placebo, 25(OH)D concentrations increased, tPTH decreased by 9% ($P < 0.001$), n-oxPTH by 7% ($P = 0.025$) and the ratio of n-oxPTH/tPTH increased ($P = 0.027$). Changes in phosphate and HDL concentration correlated with changes in n-oxPTH, but not tPTH.

Conclusions: tPTH and n-oxPTH decrease upon vitamin D supplementation. Our study suggests that vitamin D supplementation reduces the oxidation of PTH, as we observed a small but significant increase in the non-oxidised proportion of PTH upon treatment. In addition, we found that changes in phosphate and HDL concentration showed a relationship with changes in n-oxPTH, but not tPTH. This may be explained by the biological activity of n-oxPTH. Further research should be carried out to establish the clinical relevance of n-oxPTH.

Key Words

- ▶ biochemical markers of bone turnover
- ▶ parathyroid hormone
- ▶ non-oxidised PTH
- ▶ randomised clinical trial
- ▶ vitamin D

Endocrine Connections
(2019) **8**, 518–527

Introduction

Parathyroid hormone (PTH) plays a critical role in maintaining adequate serum calcium homeostasis. It increases serum calcium by stimulating bone resorption, promoting phosphate excretion, converting vitamin D to its active form and by limiting calcium excretion. The PTH molecule can be oxidised at methionine residues 8 and 18 (1). This results in an inability to activate the PTH-1 receptor, rendering the hormone biologically inactive (1, 2, 3, 4). The oxidation of PTH occurs only *in vivo* and does not progress after blood withdrawal (5).

Over the past decades, PTH assays have improved greatly. The first-generation PTH assays were radioimmunoassays and used a single antibody to quantify PTH levels in the blood. However, these assays measured not only intact PTH, but also PTH fragments with no biological activity. Subsequently, a second-generation immunoassay was developed, using the sandwich principle with two antibodies directed close to the C- and N-terminal parts. This resulted in a major improvement in diagnostic and prognostic use of PTH measurements. A third-generation assay captured the full-length PTH and had antibodies detected against the most proximal and distal binding sites of the hormone (6). There is still an ongoing debate on whether these third-generation assays have additional value compared to the second-generation assays. However, not only fragmentation, but also the above-mentioned posttranslational oxidation of PTH can affect the measurements (7). The inactive oxidised PTH (oxPTH) cross-reacts in the frequently used PTH immunoassays (3, 4). Therefore, the assumption that the PTH measured by the third generation is biologically active PTH, is probably not the case, as this assay measured both the oxidised or non-oxidised PTH.

A newly developed method enables measurement of solely the non-oxidised fraction of PTH (8). This non-oxidised PTH (n-oxPTH) can be measured by automated immunoassays in the eluate which remains after removal of oxidised PTH by using an oxPTH affinity column.

As only n-oxPTH activates the PTH-1 receptor, it may reflect the hormonal function of PTH better than the currently measured PTH, which is a combination of oxidised and non-oxidised PTH. As a large fraction of circulating PTH is oxidised, this total PTH (tPTH) may rather reflect oxidative stress-related pathology (9). Tepel *et al.* showed that haemodialysis patients with higher n-oxPTH concentrations had an increased survival. Per contra, tPTH is associated with mortality in both chronic kidney disease patients and in healthy individuals (10, 11).

In addition, Seiler-Mussler *et al.* recently concluded that currently measured PTH is associated with all-cause mortality, in contrast to n-oxPTH in chronic kidney disease (12). This opposite relationship supports the rationale that n-oxPTH may reflect the hormonal status more accurately.

PTH secretion is suppressed by 1,25(OH)₂Vitamin D and to a lesser extent by 25(OH)Vitamin D (25(OH)D) (13). Hence, 25(OH)D deficiency leads to increased PTH levels in order to prevent deranging calcium homeostasis. Conversely, PTH secretion is diminished after vitamin D supplementation.

In this study, we aimed to investigate the effect of vitamin D supplementation on the n-oxPTH concentrations in comparison to tPTH in a population with normal renal function. For this purpose, we measured n-oxPTH in the Styrian Hypertension Study, a vitamin D randomised controlled trial (14). In addition, we investigated the relationship between bone resorption and formation markers, markers of lipid metabolism and parameters of mineral metabolism and oxidative stress with n-oxPTH and tPTH.

Subjects and methods

Study design and participants

Samples from the Styrian Vitamin D Hypertension Trial, a single-centre, double-blind, placebo-controlled, parallel-group study performed at the Medical University of Graz, Austria (NCT02136771; ClinicalTrials.gov), with sufficient material for n-oxPTH measurements were used (14).

The participants were aged 18 years or older, were diagnosed with arterial hypertension and had 25(OH) D concentrations <75 nmol/L (according to the assay used in the trial: IDS-iSYS 25-hydroxyvitamin assay; Immunodiagnostic Systems Ltd., Boldon, UK). Arterial hypertension was defined as an office blood pressure of systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg, a mean 24-h ambulatory blood pressure monitoring of systolic ≥ 125 mmHg or diastolic ≥ 80 mmHg, a home blood pressure of systolic ≥ 130 mmHg or diastolic ≥ 85 mmHg or ongoing antihypertensive treatment. For study details, we refer to the article published by Pilz *et al.* (14). Exclusion criteria included hypercalcaemia, an estimated glomerular filtration rate <15 mL/min per 1.73 m², a regular intake of >880 IU of vitamin D daily during the last 4 weeks in addition to the study medication, 24-h systolic blood pressure >160 mmHg or <120 mmHg, 24-h diastolic blood pressure >100 mmHg, pregnant or lactating women,

acute coronary syndrome, cerebrovascular events within the previous 2 weeks, diseases with an estimated life expectancy of less than 1 year, any clinically significant acute disease requiring drug treatment, chemotherapy or radiation therapy and a change in antihypertensive treatment during the previous 4 weeks or planned change of antihypertensive treatment.

Written informed consent was obtained from all study participants. The study was approved by the Ethics Committee of the Medical University of Graz, Austria and was designed to comply with the Declaration of Helsinki.

Intervention

Eligible participants were randomly assigned to receive either 2800 IU of vitamin D₃ (Oleovit D₃, Fresenius Kabi Austria, Austria) or a matching placebo administered orally by seven oily drops per day for 8 weeks. Randomisation was carried out using Web-based randomisation software (Randomizer). All investigators and authors enrolling participants, collecting data and assigning intervention were masked to participant allocation. The study took place between June 2011 and August 2014. Detailed information was published previously (14).

Outcome measures

In this *post hoc* study analysis, we aimed to investigate the effects of vitamin D supplementation on tPTH and n-oxPTH concentrations between groups, adjusted for baseline values. In addition, we studied the correlation between n-oxPTH, tPTH concentrations, markers of bone turnover, lipid metabolism, calcium and phosphate homeostasis and asymmetric dimethylarginine (ADMA), a downstream marker of oxidative stress

Biochemical analysis

EDTA plasma samples were obtained after an overnight fast and were centrifuged and stored at -80°C until determination. $25(\text{OH})\text{D}_3$ was assessed by isotope dilution liquid chromatography-tandem mass spectrometry at the Endocrine Laboratory of the VU University Medical Center, as described previously (15). The limit of quantitation was 1.2 nmol/L and the intra- and inter-assay variation (CV) was 3 and 6%, respectively. PTH concentration was measured in EDTA plasma using an automated second-generation PTH immunoassay (Elecsys, Roche Diagnostics) with an intra- and inter-assay CV of <2.7 and <6.5%, respectively. N-oxPTH concentration

was determined using an oxPTH affinity column (A1112; Immundiagnostik AG, Bensheim, Germany). These affinity columns are filled with an antibody containing slurry that specifically binds the oxidised form of PTH and after being centrifuged, the eluate contains only the non-oxidised form of PTH (8). The columns were filled with 300 μL EDTA plasma and incubated end-over-end at room temperature for 1 h. Afterwards, we determined n-oxPTH in the eluate using a second-generation PTH immunoassay (Elecsys, Roche Diagnostics). Combined with use of the n-oxPTH columns the inter-assay CV <2 pmol/L and >2 pmol/L is 10 and 2.4%, respectively (5).

Total osteocalcin (OC), a bone formation marker, was measured by an electrochemiluminescence immunoassay (Elecsys, Roche Diagnostics). The intra- and inter-assay CV was 0.5 and 1.4%, respectively. CTX, a bone resorption marker, was measured by electrochemiluminescence immunoassay (Elecsys, Roche Diagnostics). The intra- and inter-assay CV was 2.0 and 4.2%, respectively. Concentrations of bone-specific alkaline phosphatase, also a marker of bone formation (bALP; inter-assay CV: 5.2%), were determined by a spectrophotometric immunoassay (IDS-ISYS Ostase BAP; Immunodiagnostic Systems Ltd. (IDS Ltd.), Boldon, Tyne & Wear, UK). Procollagen type 1 amino-terminal propeptide (PINP), also a bone formation marker, was measured by an automated electrochemiluminescence immunoassay (Elecsys, Roche Diagnostics) with an inter-assay CV of 2.7%. To measure $1,25(\text{OH})_2\text{D}_3$, a chemiluminescence immunoassay (IDS-iSYS 1,25VitDXp; Immunodiagnostic Systems Ltd., Boldon, UK) was used, of which the intra- and inter-assay CV was 6.4–12.1% and 6.6–9.6%, respectively. Vitamin D-binding protein was measured by the Quantikine Human Vitamin D-Binding Protein immunoassay (R&D Systems, Inc.). Intra- and inter-assay coefficients of variation were <5.1 and <7.4%, respectively. FGF23 was measured by a multi-matrix ELISA (FGF23 (C-terminal) ELISA; Biomedica Medizinprodukte GmbH & CO KG, Vienna, Austria). The intra-assay and inter-assay coefficients of variation (CVs) were ≤ 12 and $\leq 10\%$, respectively. The effect of vitamin D supplementation on bone turnover markers and FGF23 in this cohort was published previously (16, 17). Total cholesterol, HDL and triglycerides were measured using an enzymatic colorimetric assay (Elecsys, Roche Diagnostics). LDL was calculated according to the Friedewald equation. Asymmetric dimethylarginine (ADMA), a downstream marker of oxidative stress, was measured with reversed-phase HPLC (18). Assay specifications and the effect of vitamin D supplementation on ADMA in this cohort were published previously (19).

Statistical analysis

Data following a normal distribution are shown as means with standard deviation (s.d.), data following a skewed distribution are shown as medians with interquartile ranges. Skewed variables were log transformed prior to parametric analyses. At baseline, the Mann–Whitney *U* test was used to assess the differences between the vitamin D-treated group and placebo-treated group. In order to compare the decrease in n-oxPTH with the decrease in tPTH, the Mann–Whitney *U* test was also used between the percentage differences. Pearson's and Spearman's correlation coefficients were used to evaluate the relationship between baseline parameters, n-oxPTH and tPTH. Spearman's correlation coefficient was used when skewed variables were not normally distributed after log transformation. In addition, Spearman's correlation coefficient was used to assess the correlation between the change, calculated as the difference between the parameter measured at the final study visit and the same parameter at the baseline study visit, in n-oxPTH, tPTH and the n-oxPTH/tPTH ratio and several parameters in the vitamin D-treated group. These calculated changes are marked with 'Δ'. Also, Bonferroni corrected values were reported. Analysis of covariance (ANCOVA) with adjustment for baseline values was used to test for differences in the outcome variables (i.e. 25(OH)D₃, n-oxPTH, tPTH, plasma calcium, 24-h calcium excretion, 1,25(OH)₂D, biologically available 25(OH)D₃, the n-oxPTH/tPTH ratio and ADMA). Values more than 3SD from the mean were deemed as outliers, they were removed and the analysis repeated. This is marked in the results section. Biologically available 25(OH)D₃ was calculated using equations adapted from Vermeulen *et al.* (20). For the adapted formula and an example of the calculation of bioavailable 25(OH)D, we refer to the supplement of Powe *et al.* (21). The percentage change of a given parameter in the vitamin D-treated group was calculated by dividing the change from baseline after treatment by its respective baseline value.

A *P* value <0.05 was considered statistically significant. All analyses were performed using SPSS version 22 (SPSS).

Results

The trial was completed by 188 participants; the participant flow chart was published previously (14). For the current study, we included 108 participants (53 in treatment group and 55 in placebo group) of which

sufficient material was present for n-oxPTH measurement at baseline and follow-up. The baseline characteristics of the included patients are shown in Table 1. There was no significant difference between the vitamin D- and placebo-treated group at baseline.

Cross-sectional analysis

The Pearson correlation coefficients between n-oxPTH, tPTH, bone formation markers (bALP, osteocalcin, P1NP), a bone resorption marker (CTX) and FGF23 are shown in Table 2. Spearman correlation analysis for assessing non-linear relationships are shown in Supplementary Table 1 and Supplementary Table 2 (see section on supplementary data given at the end of this article); these did not considerably differ from the results of the Pearson correlation analysis. tPTH and n-oxPTH showed a significant correlation ($r=0.555$; $P<0.001$; Fig. 1). Bone formation or resorption markers and FGF23 did not exhibit a significant correlation with either tPTH or n-oxPTH at baseline after Bonferroni adjustment. Table 2 also displays that total cholesterol, HDL, LDL and triglycerides showed no significant correlation with n-oxPTH or tPTH after Bonferroni adjustment.

The relationship between mineral metabolism markers, ADMA and vitamin D and tPTH and n-oxPTH is shown in Table 3. After Bonferroni correction, none of the tested parameters showed a significant correlation.

Interventional analysis

After vitamin D treatment, ANCOVA analysis revealed for 25(OH)D₃ concentrations a mean treatment effect (95 % CI) of 32.4 nmol/L (25.9–38.8); $P<0.001$. This can be seen in Table 4. Biologically available 25(OH)D also increased compared to the placebo group (treatment effect: 3.91 nmol/L (2.64–5.18); $P<0.001$). Both tPTH and n-oxPTH decreased after vitamin D treatment (−0.90 (−0.40 to −1.40) pmol/L; $P<0.001$; and −0.08 (−0.01 to −0.15) pmol/L; $P=0.025$, respectively). The decrease in tPTH and n-oxPTH (9 and 7%, respectively) was not significantly different ($P=0.51$). The ratio of n-oxPTH/tPTH showed a trend for increase after vitamin D treatment (0.022 (0.003–0.042); $P=0.027$ or expressed as percentage by 2.2% (0.3–4.2%)) In addition, 1,25(OH)₂D concentrations increased after treatment (10.6 pg/mL (3.94–17.2); $P=0.002$), while plasma calcium concentrations and 24-h urinary calcium excretion were not affected. ADMA did not show a significant treatment effect after vitamin D supplementation.

Table 1 Baseline characteristics of participants in the vitamin D ($n = 53$) and placebo ($n = 55$)-treated groups.

Parameters	All	N	Vitamin D	N	Placebo	N	P value
Age (years)	60.1 ± 11.9	108	59.5 ± 11.8	53	60.7 ± 12.1	55	0.384
Gender (% female)	60	108	29	53	31	55	0.863
% postmenopausal females	47	59	24	29	23	30	0.561
BMI (kg/m ²)	30.0 ± 5.3	107	30.3 ± 4.9	53	29.7 ± 5.8	54	0.501
25(OH)D ₃ (nmol/L)	46 (25–58)	107	46 (37–58)	52	46 (30–59)	54	0.538
1,25(OH) ₂ D (pg/mL)	48 (35–65)	107	49 (38–66)	53	42 (29–64)	54	0.153
Vitamin D binding protein (µg/mL)	249 ± 110	107	237 ± 108	53	258 ± 112	54	0.355
Bioavailable 25(OH)D ₃ (nmol/L)	5.80 (3.76–8.13)	106	6.60 (4.10–8.03)	52	5.15 (3.20–8.47)	54	0.123
Serum calcium (mmol/L)	2.26 (2.21–2.33)	108	2.26 (2.21–2.33)	53	2.26 (2.21–2.33)	55	0.824
Serum phosphate (mmol/L)	0.94 ± 0.17	108	0.92 ± 0.16	53	0.96 ± 0.17	55	0.199
24 h urinary calcium excretion (mmol/24 h)	3.34 (1.85–5.01)	97	3.63 (1.88–6.23)	48	2.96 (1.85–4.86)	49	0.325
N-oxPTH (pmol/L)	1.1 ± 0.3	108	1.1 ± 0.3	53	1.1 ± 0.3	55	0.340
Total PTH (pmol/L)	5.3 (4.0–6.7)	108	5.2 (4.1–6.7)	53	5.5 (3.9–6.8)	55	0.710
bALP (µg/L)	17 (13–21)	103	17 (13–21)	51	17 (13–20)	52	0.962
CTX (ng/mL)	0.19 (0.11–0.33)	100	0.19 (0.13–0.34)	50	0.18 (0.10–0.28)	50	0.647
Osteocalcin (ng/mL)	13 (10–19)	106	13 (10–18)	52	14 (9–19)	54	0.859
P1NP (ng/mL)	38 (31–52)	103	38 (29–56)	50	38 (32–49)	53	0.879
eGFR CKD-EPI (mL/min/1.73 m ²)	83 ± 19	108	85 ± 18	53	81 ± 19	55	0.336
FGF23 (pmol/L)	0.83 (0.59–1.24)	107	0.77 (0.54–1.17)	53	0.89 (0.69–2.02)	54	0.069
Office systolic BP* (mmHg)	145 ± 16	107	145 ± 17	53	145 ± 16	54	0.856
Office diastolic BP* (mmHg)	87 ± 11	107	87 ± 10	53	87 ± 11	54	0.981
Ratio n-oxPTH/tPTH	0.22 ± 0.07	108	0.22 ± 0.07	53	0.22 ± 0.06	55	0.850
Total cholesterol (mg/dL)	192 ± 40	108	198 ± 39	53	186 ± 40	55	0.104
HDL (mg/dL)	55.5 (47–66)	108	54.0 (45–66)	53	56.0 (47–66)	55	0.612
LDL (mg/dL)	112 ± 35	105	117 ± 33	53	107 ± 37	52	0.137
Triglycerides (mg/dL)	110 (69–153)	108	109 (69–152)	53	110 (69–162)	55	0.996
ADMA (µmol/L)	0.70 (0.63–0.78)	108	0.69 (0.63–0.78)	53	0.71 (0.64–0.79)	55	0.247

Data are shown as mean ± s.d. or median (IQR).

*Maximum of the two arms.

Correlation analysis was also applied to test whether the changes in several parameters before and after vitamin D supplementation in the vitamin D-treated group were correlated with Δ n-oxPTH, Δ tPTH and the Δ n-oxPTH/tPTH ratio in the vitamin D-treated group and several other parameters. These results are presented in Table 5. What is apparent from these data is that

the difference in serum phosphate exhibited a significant, inverse correlation with Δ n-oxPTH, but not with Δ tPTH ($\rho = -0.418$; $P = 0.002$; Bonferroni adjusted: $P = 0.020$ and $\rho = -0.314$; $P = 0.022$; Bonferroni adjusted: $P = 0.264$, respectively). Also the Δ HDL correlated with Δ n-oxPTH, but not Δ tPTH ($\rho = 0.499$; $P < 0.001$; Bonferroni adjusted: $P = 0.002$ and $\rho = 0.079$; $P = 0.576$;

Table 2 Correlations between bone turnover parameters, parameters of lipid metabolism and n-oxPTH or tPTH.

Parameters	n-oxPTH			Total PTH*		
	Pearson's r	P value	Bonferroni adjusted P value	Pearson's r	P value	Bonferroni adjusted P value
Total PTH*	0.555	<0.001	<0.001	n.a.	n.a.	n.a.
bALP*	0.144	0.148	1.000	0.024	0.813	1.000
CTX*	-0.028	0.783	1.000	-0.060	0.552	1.000
Osteocalcin*	0.237	0.014	0.252	0.108	0.268	1.000
P1NP*	0.169	0.088	1.000	-0.029	0.774	1.000
FGF23*	-0.016	0.869	1.000	0.010	0.916	1.000
Total cholesterol	-0.047	0.625	1.000	0.008	0.934	1.000
HDL*	0.254	0.008	0.144	0.166	0.085	1.000
LDL	-0.089	0.365	1.000	-0.021	0.831	1.000
Triglycerides*	-0.216	0.025	0.450	-0.042	0.666	1.000

*Log transformed.

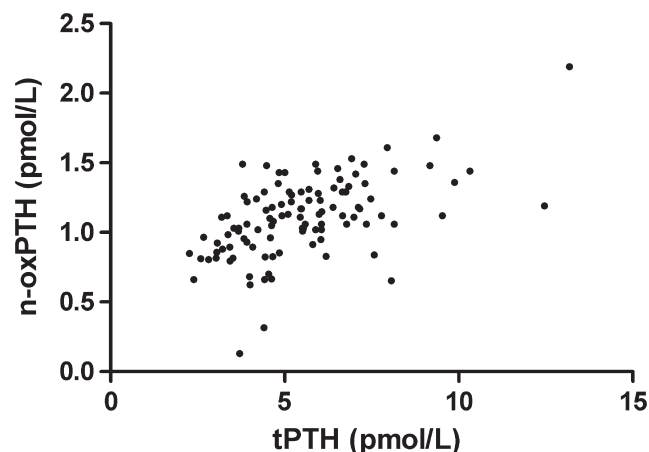


Figure 1 Scatterplot of tPTH and n-oxPTH at baseline ($n = 108$). Pearson's $r = 0.555$; $P < 0.001$.

Bonferroni adjusted: $P = 1.0$, respectively). Δ n-oxPTH/tPTH ratio did not show a significant correlation with the tested parameters.

Discussion

We determined the effect of vitamin D supplementation on n-oxPTH concentrations in an RCT in hypertensive vitamin D-insufficient patients with preserved kidney function and found a significant decrease in n-oxPTH concentration during supplementation. Moreover, the correlation between n-oxPTH and tPTH at baseline was only 0.555 ($P < 0.001$), indicating large individual differences of oxidised PTH in patients with preserved renal function, as can also be seen in Fig. 1.

N-oxPTH is presumed to be the biologically active form of PTH and may reflect the hormonal activity in a

more accurate way, since oxidised PTH does not activate the PTH-1 receptor (22). Upon vitamin D treatment, both tPTH and n-oxPTH concentrations decreased. Interestingly, tPTH decreased more strongly than n-oxPTH. This resulted in a significantly increased ratio of n-oxPTH/tPTH upon supplementation. This indicates that vitamin D supplementation results in an increase in the non-oxidised fraction of PTH, or stated otherwise, that the oxidised proportion is reduced (Fig. 2). As the clinical relevance of this finding is yet to be established and the change in the n-oxPTH/tPTH ratio is small, no vast conclusions can be drawn. However, hypothetically this would implicate that vitamin D treatment leads to a reduction in the oxidation of PTH.

Caution must be applied when interpreting this ratio. We previously published that this ratio cannot reflect the true non-oxidised percentage of the tPTH, as there is no standard for n-oxPTH yet (5). Calculation of this ratio also showed poor agreement between several immunoassays. Although the n-oxPTH measurements are validated extensively, the ratio over tPTH, and hence, the percentage PTH that is free of oxidation, differs between several immunoassays. For this reason, we can measure n-oxPTH reliably; however, the ratio can only be used to assess changes over time.

There is a growing body of evidence about the protective effect of vitamin D on oxidative stress. Antioxidant properties of vitamin D are shown *in vitro* and *in vivo*, in rats as well as in humans (23, 24, 25, 26). PTH oxidation may, therefore, also be influenced by vitamin D supplementation, as is suggested by our data. However, there was no significant treatment effect on ADMA, a downstream marker of oxidative stress, in this study. (18, 19)

PTH stimulates bone resorption. Osteoblasts express the PTH-1 receptor, which is activated by PTH. Once activated,

Table 3 Correlations between parameters of calcium and phosphate homeostasis, ADMA and n-oxPTH or tPTH.

Parameters	n-oxPTH			Total PTH*		
	Pearson's r	P value	Bonferroni adjusted			
			P value	P value	P value	
25(OH)D ₃ *	-0.069	0.477	1.000	-0.056	0.564	1.000
Bioavailable 25(OH)D ₃ *	-0.062	0.526	1.000	-0.111	0.258	1.000
1,25(OH) ₂ D*	0.179	0.065	1.000	0.215	0.026	0.572
eGFR	0.080	0.410	1.000	-0.063	0.519	1.000
Calcium	-0.053	0.588	1.000	0.008	0.935	1.000
Phosphate	-0.114	0.239	1.000	-0.213	0.027	0.594
UPCR	0.088	0.363	1.000	0.183	0.059	1.000
24-h urinary calcium†	0.063	0.542	1.000	-0.127	0.216	1.000
ADMA*	-0.104	0.285	1.000	0.017	0.861	1.000

*Log transformed; †Spearman's rho.

Bioavailable 25(OH)D₃, biologically available 25(OH)D₃; UPCR, urinary phosphate to creatinine ratio.

Table 4 ANCOVA for the effect of vitamin D or placebo treatment.

Parameters	Group	Baseline (SD/IQR)	Follow-up (SD/IQR)	Mean change (95% CI)	Treatment effect (95% CI)	P value
25(OH)D ₃ (nmol/L)	Vitamin D, N = 52	49.0 ± 18.1	79.3 ± 19.1	30.3 (24.1–36.4)	32.4 (25.9–38.8)	<0.001
	Placebo, N = 54	46.0 ± 18.5	45.3 ± 19.7	−0.72 (−4.77 to 3.32)		
Total PTH (pmol/L)*	Vitamin D, N = 53	5.19 (4.13–6.69)	4.64 (3.90–5.80)	−0.61 (−0.96 to −0.27)	−0.90 (−0.40 to −1.40)	<0.001
	Placebo, N = 55	5.46 (3.92–6.66)	5.33 (4.07–7.07)	0.35 (−0.03 to 0.74)		
n-oxPTH (pmol/L)	Vitamin D, N = 53	1.1 ± 0.3	1.1 ± 0.3	−0.08 (−0.13 to −0.03)	−0.08 (−0.01 to −0.15)	0.025
	Placebo, N = 55	1.1 ± 0.3	1.1 ± 0.3	0.01 (−0.04 to 0.07)		
Plasma calcium (mmol/L)	Vitamin D, N = 53	2.28 ± 0.10	2.27 ± 0.09	−0.01 (−0.03 to 0.02)	0.02 (−0.01 to 0.05)	0.294
	Placebo, N = 55	2.27 ± 0.11	2.25 ± 0.11	−0.01 (−0.03 to 0.01)		
24-h urinary calcium excretion (mmol/24 h)*	Vitamin D, N = 37	3.60 (1.45–6.25)	3.80 (1.80–6.40)	0.43 (−0.14 to 1.00)	0.66 (−0.07 to 1.39)	0.077
	Placebo, N = 40	2.95 (1.90–5.40)	3.00 (1.50–4.60)	−0.19 (−0.67 to 0.29)		
1,25(OH) ₂ D (pg/mL)	Vitamin D, N = 53	53.5 ± 20.9	60.2 ± 25.1	6.66 (1.06–12.3)	10.6 (3.94–17.2)	0.002
	Placebo, N = 53	47.0 ± 20.9	45.3 ± 15.9	−1.12 (−5.84 to 3.59)		
Vitamin D binding protein (µg/mL)	Vitamin D, N = 52	239 ± 108	323 ± 319	85 (−6 to 177)	12 (−88 to 111)	0.816
	Placebo, N = 54	258 ± 112	315 ± 173	54 (−4 to 112)		
Bioavailable 25(OH)D ₃ (nmol/L)*,†	Vitamin D, N = 50	6.60 (4.08–8.01)	8.64 (6.57–12.0)	2.25 (0.90–3.60)	3.91 (2.64–5.18)	<0.001
	Placebo, N = 53	5.22 (3.15–8.51)	4.98 (2.94–6.87)	−1.30 (−2.66 to 0.05)		
Ratio n-oxPTH/tPTH	Vitamin D, N = 53	0.22 ± 0.07	0.23 ± 0.08	0.009 (−0.009 to 0.027)	0.022 (0.003 to 0.042)	0.027
	Placebo, N = 55	0.22 ± 0.06	0.20 ± 0.05	−0.01 (−0.025 to 0.001)		
ADMA*,† (µmol/L)	Vitamin D, N = 53	0.69 (0.63–0.77)	0.73 (0.65–0.81)	0.04 (0.01–0.07)	0.008 (−0.028 to 0.045)	0.759
	Placebo, N = 54	0.72 (0.65–0.79)	0.75 (0.67–0.80)	0.02 (−0.01 to 0.05)		

Data are shown as mean ± s.d. or median (IQR).

*Skewed variables for which transformed values were used in ANCOVA, but untransformed values are shown in the table; †1 outlier was excluded; this had no effect on the significance level of the analysis.

osteoblasts increase their expression of RANK and inhibit the secretion of osteoprotegerin, resulting in the stimulation of osteoclast differentiation. Although PTH stimulates bone resorption, the level of PTH does not reflect bone turnover accurately. In patients with chronic kidney disease (CKD) with increasing PTH levels, the PTH concentration does not correlate well with bone turnover, based on bone histomorphometry (27). The presumed mechanism is end-organ resistance and possibly also

posttranslational oxidation of PTH (28). In addition, a recent study showed that not only the PTH molecule, but also the PTH receptor can be oxidised (29). In this study, Ardura *et al.* used modified PTH (1-34), where methionine residues 8 and 18 were replaced with norleucine. This way, the two residues that are prone to oxidation were stabilised. They incubated cells with H₂O₂ and concluded that this reduced all PTH-dependent signalling pathways. In consequence, both oxidation of the PTH molecule and the receptor could contribute to the limited correlation between PTH and bone histomorphometry in individuals with increased oxidative stress.

PTH is known to lead to decreased reabsorption of phosphate, decreased excretion of calcium and stimulation of 1-alpha-hydroxylase in the kidney. In our study, baseline concentrations of n-oxPTH and tPTH were not associated with phosphate concentrations. Interestingly, however, in the vitamin D group, the change in (Δ) phosphate did not show a correlation with ΔtPTH, but did show a significant correlation with Δn-oxPTH (Table 5). This lack of a relationship between phosphate and tPTH dynamics, in contrast to a significant relationship between phosphate and n-oxPTH dynamics may be explained by the biological activity of n-oxPTH.

N-oxPTH and tPTH did not correlate with measured bone turnover makers, which does not suggest an important role of measuring n-oxPTH for determining bone turnover in our population. Yet, the setting in our

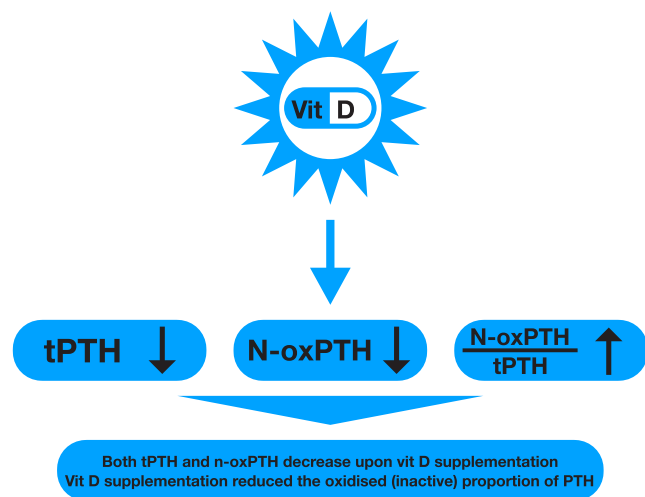


Figure 2

Vitamin D supplementation decreased tPTH and n-oxPTH, while the proportion of oxidised PTH was reduced.

Table 5 Correlations for changes in n-oxPTH, PTH, n-oxPTH/tPTH ratio and changes in several parameters for the vitamin D group.

Parameters	Δ n-oxPTH		Δ Total PTH		Δ n-oxPTH/tPTH ratio	
	Spearman's ρ	Bonferroni adjusted P-value	Spearman's ρ	Bonferroni adjusted P-value	Spearman's ρ	Bonferroni adjusted P-value
Δ n-oxPTH	n.a.	n.a.	0.448	0.001	0.241	0.082
Δ 25(OH)D ₃	0.008	1.000	0.043	0.764	-0.016	0.910
Δ Calcium	-0.335	0.155	-0.312	0.023	0.051	0.718
Δ Phosphate	-0.418	0.020	-0.314	0.022	0.032	0.821
Δ BAP	0.117	1.000	0.010	0.944	0.076	0.599
Δ FGF23	-0.263	0.631	-0.141	0.315	-0.030	0.828
Δ ADMA	0.095	1.000	-0.016	0.908	-0.057	0.685
Δ LDL	0.150	1.000	-0.039	0.782	0.088	0.533
Δ HDL	0.499	0.002	0.079	0.576	0.216	0.120
Δ Total cholesterol	0.194	1.000	-0.124	0.377	0.197	0.158

study might not be an optimal model to study these mechanisms and future studies in patients with renal failure are needed.

While some research has been carried out on the interrelation between plasma PTH and parameters of lipid metabolism, it is not completely understood. It seems that high concentrations of PTH, as seen in primary and secondary hyperparathyroidism (HPT), are accompanied by changes in parameters of lipid metabolism (30, 31). In the case of secondary HPT, these changes can be caused by many factors (e.g. the consequences of nephrotic syndrome), while in primary HPT, a more causal relationship seems plausible. Although study results are inconclusive, an improvement in lipid profile is frequently observed after parathyroidectomy in primary HPT (31, 32). The relation between HDL and n-oxPTH is of particular interest, as HDL has anti-oxidative properties (33). It is therefore interesting that we showed in our study a significant association between the change in n-oxPTH and the change in HDL after vitamin D treatment, which was not the case for the change in tPTH. A higher HDL concentration, and hence a better antioxidant status, could explain the positive trend with n-oxPTH. A greater focus on the oxidised status of PTH could produce interesting findings that might account for inconsistencies in earlier studies on PTH and lipid metabolism.

Limitations of this study include the relatively short treatment duration of 8 weeks, the use of a specific patient group (hypertensive patients) and the small number of patients with severe vitamin D deficiency. Hypertension itself can contribute to oxidative stress, which may also increase the proportion of oxidised PTH and lower n-oxPTH concentrations (34). Hence, the effect we observed on n-oxPTH could be an underestimation. Concerning the assessment of the correlation between n-oxPTH and bone turnover markers, our study population may not be ideal, individuals in this population are not expected to have a widely divergent rate of bone turnover. In addition, vitamin D deficiency was defined by measurements of baseline total 25(OH)D and not free 25(OH)D. Some researchers suggest that free 25(OH)D is a better marker for assessing vitamin D status (35). Also caution of interpretation is warranted, as this is a *post hoc* analysis. Notwithstanding these limitations, the RCT design and effective vitamin D treatment as well as ample inclusion of parameters are the key strengths of the current study.

In conclusion, we showed that both tPTH and n-oxPTH decrease upon vitamin D supplementation. Our study suggests that vitamin D supplementation affects the oxidation of PTH, as we observed a small but significant

increase in the non-oxidised proportion of PTH upon treatment. In addition, we found that changes in phosphate and HDL concentration showed a relationship with changes in n-oxPTH, but not tPTH. This may be explained by the biological activity of n-oxPTH. Further research should be carried out to establish the clinical relevance of n-oxPTH.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-19-0097>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The Styrian Vitamin D Hypertension Trial was supported by funding from the Austrian National Bank (Jubilaeumsfond: project no.: 13878 and 13905). Immundiagnostik AG (Bensheim, Germany) provided the oxPTH affinity columns (A1112) and funded the n-oxPTH measurements. V Francic was supported by the PhD programme Molecular Medicine at the Medical University of Graz, Graz, Austria.

Acknowledgments

The authors thank all study participants, Immundiagnostik AG and also Fresenius Kabi for providing the study medication.

References

- Zull JE, Smith SK & Wiltshire R. Effect of methionine oxidation and deletion of amino-terminal residues on the conformation of parathyroid hormone. Circular dichroism studies. *Journal of Biological Chemistry* 1990 **265** 5671–5676.
- Vogt W. Oxidation of methionyl residues in proteins: tools, targets, and reversal. *Free Radical Biology and Medicine* 1995 **18** 93–105. ([https://doi.org/10.1016/0891-5849\(94\)00158-G](https://doi.org/10.1016/0891-5849(94)00158-G))
- Galceran T, Lewis-Finch J, Martin KJ & Slatopolsky E. Absence of biological effects of oxidized parathyroid hormone-(1-34) in dogs and rats. *Endocrinology* 1984 **115** 2375–2378. (<https://doi.org/10.1210/endo-115-6-2375>)
- Horiuchi N. Effects of oxidation of human parathyroid hormone on its biological activity in continuously infused, thyroparathyroidectomized rats. *Journal of Bone and Mineral Research* 1988 **3** 353–358. (<https://doi.org/10.1002/jbmr.5650030316>)
- Ursem SR, Vervloet MG, Hillebrand JJG, De RT & Heijboer AC. Oxidation of PTH: in vivo feature or effect of preanalytical conditions? *Clinical Chemistry and Laboratory Medicine* 2018 **56** 249–255. (<https://doi.org/10.1515/ccm-2017-0313>)
- Souberbielle JCP, Roth H & Fouque DP. Parathyroid hormone measurement in CKD. *Kidney International* 2010 **77** 93–100. (<https://doi.org/10.1038/ki.2009.374>)
- Hocher B & Zeng S. Clear the fog around parathyroid hormone assays: what do iPTH assays really measure? *Clinical Journal of the American Society of Nephrology* 2018 **13** 524–526. (<https://doi.org/10.2215/CJN.01730218>)
- Hocher B, Armbruster FP, Stoeva S, Reichetzedler C, Grön HJ, Lieker I, Khadzhynov D, Slowinski T & Roth HJ. Measuring parathyroid hormone (PTH) in patients with oxidative stress – do we need a fourth generation parathyroid hormone assay? *PLoS ONE* 2012 **7** e40242. (<https://doi.org/10.1371/journal.pone.0040242>)
- Tepel M, Armbruster FP, Grön HJ, Scholze A, Reichetzedler C, Roth HJ & Hocher B. Nonoxidized, biologically active parathyroid hormone determines mortality in hemodialysis patients. *Journal of Clinical Endocrinology and Metabolism* 2013 **98** 4744–4751. (<https://doi.org/10.1210/jc.2013-2139>)
- Tentori F, Blayney MJ, Albert JM, Gillespie BW, Kerr PG, Bommer J, Young EW, Akizawa T, Akiba T, Pisoni RL, *et al.* Mortality risk for dialysis patients with different levels of serum calcium, phosphorus, and PTH: the dialysis outcomes and practice patterns study (DOPPS). *American Journal of Kidney Diseases* 2008 **52** 519–530. (<https://doi.org/10.1053/j.ajkd.2008.03.020>)
- Hagström E, Hellman P, Larsson TE, Ingelsson E, Berglund L, Sundström J, Melhus H, Held C, Lind L, Michaëlsson K, *et al.* Plasma parathyroid hormone and the risk of cardiovascular mortality in the community. *Circulation* 2009 **119** 2765–2771. (<https://doi.org/10.1161/CIRCULATIONAHA.108.808733>)
- Seiler-Mussler S, Limbach AS, Emrich IE, Pickering JW, Roth HJ, Fliser D & Heine GH. Association of nonoxidized parathyroid hormone with cardiovascular and kidney disease outcomes in chronic kidney disease. *Clinical Journal of the American Society of Nephrology* 2018 **13** 569–576. (<https://doi.org/10.2215/CJN.06620617>)
- Ritter CS, Armbrrecht HJ, Slatopolsky E & Brown AJ. 25-Hydroxyvitamin D3 suppresses PTH synthesis and secretion by bovine parathyroid cells. *Kidney International* 2006 **70** 654–659. (<https://doi.org/10.1038/sj.ki.5000394>)
- Pilz S, Gaksch M, Kienreich K, Gröbler MR, Verheyen N, Fahrleitner-Pammer A, Treiber G, Drechsler C, Ó Hartaigh B, Obermayer-Pietsch B, *et al.* Effects of vitamin D on blood pressure and cardiovascular risk factors: a randomized controlled trial. *Hypertension* 2015 **65** 1195–1201. (<https://doi.org/10.1161/HYPERTENSIONAHA.115.05319>)
- Dirks NE, Vesper HW, van Herwaarden AE, van den JMW, Kema IP, Krabbe JG & Heijboer AC. Various calibration procedures result in optimal standardization of routinely used 25(OH)D ID-LC-MS/MS methods. *Clinica Chimica Acta* 2016 **462** 49–54. (<https://doi.org/10.1016/j.cca.2016.08.016>)
- Schwetz V, Trummer C, Pandis M, Gröbler MR, Verheyen N, Gaksch M, Zittermann A, März W, Aberer F, Lang A, *et al.* Effects of vitamin D supplementation on bone turnover markers: a randomized controlled trial. *Nutrients* 2017 **9** 1–11. (<https://doi.org/10.3390/nu9050432>)
- Trummer C, Schwetz V, Pandis M, Gröbler MR, Verheyen N, Gaksch M, Zittermann A, März W, Aberer F, Steinkellner J, *et al.* Effects of vitamin D supplementation on FGF23: a randomized-controlled trial. *European Journal of Nutrition* 2018 **58** 697–703. (<https://doi.org/10.1007/s00394-018-1672-7>)
- Frijhoff J, Winyard PG, Zarkovic N, Davies SS, Stocker R, Cheng D, Knight AR, Taylor EL, Oettrich J, Ruskovska T, *et al.* Clinical relevance of biomarkers of oxidative stress. *Antioxidants and Redox Signaling* 2015 **23** 1144–1170. (<https://doi.org/10.1089/ars.2015.6317>)
- Gröbler MR, Gaksch M, Kienreich K, Verheyen ND, Schmid J, Müllner C, Richtig G, Scharnagl H, Trummer C, Schwetz V, *et al.* Effects of vitamin D3 on asymmetric- and symmetric dimethylarginine in arterial hypertension. *Journal of Steroid Biochemistry and Molecular Biology* 2018 **175** 157–163. (<https://doi.org/10.1016/j.jsbmb.2016.12.014>)
- Vermeulen A, Verdonck L & Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 3666–3672. (<https://doi.org/10.1210/jcem.84.10.6079>)

- 21 Powe CE, Ricciardi C, Berg AH, Erdenesanaa D, Collerone G, Ankers E, Wenger J, Karumanchi SA, Thadhani R & Bhan I. Vitamin D-binding protein modifies the vitamin D-bone mineral density relationship. *Journal of Bone and Mineral Research* 2011 **26** 1609–1616. (<https://doi.org/10.1002/jbmr.387>)
- 22 Hocher B, Oberthür D, Slowinski T, Querfeld U, Schaefer F, Doyon A, Tepel M, Roth HJ, Grön HJ, Reichetzedler C, *et al.* Modeling of oxidized PTH (oxPTH) and non-oxidized PTH (n-oxPTH) receptor binding and relationship of oxidized to non-oxidized PTH in children with chronic renal failure, adult patients on hemodialysis and kidney transplant recipients. *Kidney and Blood Pressure Research* 2013 **37** 240–251. (<https://doi.org/10.1159/000350149>)
- 23 Uberti F, Lattuada D, Morsanuto V, Nava U, Bolis G, Vacca G, Squarzanti DF, Cisari C & Molinari C. Vitamin D protects human endothelial cells from oxidative stress through the autophagic and survival pathways. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** 1367–1374. (<https://doi.org/10.1210/jc.2013-2103>)
- 24 Wiseman H. Vitamin D is a membrane antioxidant ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol and tamoxifen and relevance to anticancer action. *FEBS Letters* 1993 **326** 285–288. ([https://doi.org/10.1016/0014-5793\(93\)81809-E](https://doi.org/10.1016/0014-5793(93)81809-E))
- 25 Sardar S, Chakraborty A & Chatterjee M. Comparative effectiveness of vitamin D3 and dietary vitamin E on peroxidation of lipids and enzymes of the hepatic antioxidant system in Sprague-Dawley rats. *International Journal for Vitamin and Nutrition Research* 1996 **66** 39–45.
- 26 Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, Toprak A, Yazici D, Sancak S, Deyneli O, *et al.* Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. *Journal of Clinical Endocrinology and Metabolism* 2009 **94** 4023–4030. (<https://doi.org/10.1210/jc.2008-1212>)
- 27 Garrett G, Sardiwal S, Lamb EJ & Goldsmith DJA. PTH-A particularly tricky hormone: why measure it at all in kidney patients? *Clinical Journal of the American Society of Nephrology* 2013 **8** 299–312. (<https://doi.org/10.2215/CJN.09580911>)
- 28 Vervloet MG, Brandenburg VM & CKD-MBD Working Group of ERA-EDTA. Circulating markers of bone turnover. *Journal of Nephrology* 2017 **30** 663–670. (<https://doi.org/10.1007/s40620-017-0408-8>)
- 29 Ardura JA, Alonso V, Esbrit P & Friedman PA. Oxidation inhibits PTH receptor signaling and trafficking. *Biochemical and Biophysical Research Communications* 2017 **482** 1019–1024. (<https://doi.org/10.1016/j.bbrc.2016.11.150>)
- 30 Bolland MJ, Grey AB, Gamble GD & Reid IR. Association between primary hyperparathyroidism and increased body weight: a meta-analysis. *Journal of Clinical Endocrinology and Metabolism* 2005 **90** 1525–1530. (<https://doi.org/10.1210/jc.2004-1891>)
- 31 Valdemarsson S, Lindblom P & Bergenfelz A. Metabolic abnormalities related to cardiovascular risk in primary hyperparathyroidism: effects of surgical treatment. *Journal of Internal Medicine* 1998 **244** 241–249. (<https://doi.org/10.1046/j.1365-2796.1998.00366.x>)
- 32 Ljunghall S, Lithell H, Vessby B & Wide L. Glucose and lipoprotein metabolism in primary hyperparathyroidism. Effects of parathyroidectomy. *Acta Endocrinologica* 1978 **89** 580–589. (<https://doi.org/10.1530/acta.0.0890580>)
- 33 Soran H, Schofield JD & Durrington PN. Antioxidant properties of HDL. *Frontiers in Pharmacology* 2015 **6** 222. (<https://doi.org/10.3389/fphar.2015.00222>)
- 34 Montezano AC & Touyz RM. Oxidative stress, Nox, and hypertension: experimental evidence and clinical controversies. *Annals of Medicine* 2012 **44** S2–S16. (<https://doi.org/10.3109/07853890.2011.653393>)
- 35 Tsuprykov O, Chen X, Hocher CF, Skoblo R, Yin L & Hocher B. Why should we measure free 25(OH) vitamin D? *Journal of Steroid Biochemistry and Molecular Biology* 2018 **180** 87–104. (<https://doi.org/10.1016/j.jsbmb.2017.11.014>)

Received in final form 27 March 2019

Accepted 4 April 2019

Accepted Preprint published online 4 April 2019