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Original Article

ORIGINAL ARTICLE

Intact FGF23 predicts serum phosphate improvement after combined nicotinamide and phosphate binder treatment in hemodialysis patients

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ABSTRACT

Background. Hyperphosphatemia is associated with increased mortality and cardiovascular morbidity of end-stage kidney failure (ESKF) patients. Managing serum phosphate in ESKF patients is challenging and mostly based on limiting intestinal phosphate absorption with low phosphate diets and phosphate binders (PB). In a multi-centric, double-blinded, placebo-controlled study cohort of maintenance hemodialysis patients with hyperphosphatemia, we demonstrated the efficacy of nicotinamide modified release (NAMR) formulation treatment in addition to standard PB therapy in decreasing serum phosphate. Here we aimed to assess the relationship between phosphate, FGF23, inflammation and iron metabolism in this cohort.

Methods. We measured the plasma concentrations of intact fibroblast growth factor 23 (iFGF23) and selected proinflammatory cytokines at baseline and Week 12 after initiating treatment.

Results. We observed a strong correlation between iFGF23 and cFGF23 (C-terminal fragment plus iFGF23). We identified iFGF23 as a better predictor of changes in serum phosphate induced by NAMR and PB treatment compared with cFGF23. Recursive partitioning revealed at baseline and Week 12, that iFGF23 and cFGF23 together with T50 propensity were the most important predictors of serum phosphate, whereas intact parathyroid hormone (iPTH) played a minor role in this

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model. Furthermore, we found serum phosphate and iPTH as the best predictors of iFGF23 and cFGF23. Sex, age, body mass index, and markers of inflammation and iron metabolism had only a minor impact in predicting FGF23.
Conclusion. Lowering serum phosphate in ESKF patients may depend highly on iFGF23 which is correlated to cFGF23 levels. Serum phosphate was the most important predictor of plasma FGF23 in this ESKF cohort.

LAY SUMMARY

Patients with end-stage kidney failure (ESKF) have a higher mortality and cardiovascular disease risk than the normal population in part due to hyperphosphatemia. Phosphate binders and nicotinamide help to control hyperphosphatemia in ESKF patients. Here we examined how the hormone fibroblast growth factor 23 (FGF23), phosphate, markers of iron metabolism and inflammation correlate in patients treated for 12 weeks with a novel formulation of nicotinamide. We demonstrate that intact and cleaved forms of FGF23 correlate tightly and had a similar capability to predict phosphate levels. Markers of iron metabolism or inflammation had only weak correlations with FGF23 or phosphate. Our data are consistent with an important role of FGF23 in regulating phosphate in patients with ESKF.

Keywords: FGF23, haemodialysis, hyperparathyroidism, inflammation, iron, phosphatemia

INTRODUCTION

End-stage kidney failure (ESKF) patients have a higher risk for cardiovascular disease and mortality than the normal population [1]. Hyperphosphatemia, high fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH) levels, and low calcitriol and soluble αKlotho levels in plasma are characteristic hallmarks of ESKF [2, 3]. Hyperphosphatemia in ESKF is associated with a higher mortality and cardiovascular disease risk [4, 5], and limited evidence suggests that reducing phosphate levels in ESKF patients may improve their outcome [6, 7]. Current treatment is mostly focused on reducing intestinal phosphate absorption [8]. However, nutritional management alone is insufficient to reduce serum phosphate in hemodialysis patients [9]. Thus, phosphate binders (PB) are used as standard therapy to control serum phosphate in ESKF patients [10]. Yet, as in most patients the treatment is ineffective, further drugs or combination of drugs are under investigation, such as the combination of PB with nicotinamide [11]. Recently, in the NOPHOS (Efficacy and tolerability of nicotinamide as add-on therapy compared to placebo in dialysis-dependent patients with hyperphosphatemia) study, hemodialysis patients with hyperphosphatemia (phosphate concentration >4.5 mg/dL) were treated for up to 52 weeks with PB and a nicotinamide modified release (NAMR) formulation or with PB and placebo [12, 13]. A combined therapy with PB and NAMR resulted in a significant decrease of serum phosphate and PTH after 12 and 24 weeks of treatment compared with the PB and placebo therapy. However, this effect was ceased after 52 weeks of treatment most probably due to numerous non-compliant patients. These hemodialysis patients, as expected, had very high C-terminal FGF23 (cFGF23) plasma concentration.

There is an ongoing debate about the suitability of including plasma FGF23 as marker of kidney function, kidney disease, cardiovascular disease risk or exposure to phosphate [14, 15]. The analytical variability of the current enzyme-linked immunosorbent assays (ELISA) is still an issue for incorporating FGF23 determinations in the standard testing repertoires and hinders comparison between studies [16, 17]. Moreover, several studies used cFGF23 ELISA kits, which measure total plasma FGF23 consisting of both the cleaved C-terminal FGF23 fragment and the active intact FGF23 (iFGF23) (referred to in the manuscript as cFGF23), whereas other studies measure only plasma iFGF23 with a specific iFGF23 ELISA kit. In hemodialysis patients the use of plasma cFGF23 or iFGF23, may deliver similar results as the proportion of the C-terminal fragment over the intact FGF23 peptide is low when compared with mild-stage chronic kidney disease (CKD) patients and healthy subjects [17].

Proinflammatory cytokines and iron deficiency are known stimulators of FGF23 synthesis [18–22]. Studies in ESKF patients undergoing dialysis are ambiguous and only partly show an association between plasma FGF23 and proinflammatory cytokines [23–26], whereas parameters of iron metabolism frequently are associated with plasma cFGF23 and to a lesser extent with plasma iFGF23 in CKD and hemodialysis patients as well as in the general population [27–33].

In this *post hoc* analysis of the NOPHOS cohort consisting of hemodialysis patients with hyperphosphatemia we additionally measured iFGF23 levels and selected proinflammatory cytokines in plasma samples to analyze the relationship between iFGF23 and cFGF23, to investigate the prediction capacity of iFGF23 and cFGF23 of phosphate levels and to identify predictors of iFGF23 and cFGF23 among markers of inflammation and parameters of iron metabolism.

MATERIALS AND METHODS

Study population

The NOPHOS study is a prospective, multicenter, randomized, double-blind, placebo-controlled study aiming to evaluate the efficacy and tolerability of a NAMR in combination with PB. Details on the study population and exclusion criteria have been published previously by Ketteler *et al.* [12]. Briefly, patients aged 18 years and older undergoing regular maintenance hemodialysis (3× a week) for at least 6 months prior to screening with serum phosphate concentration of >4.5 and <8.7 mg/dL, and receiving one or two oral phosphate-binding agents with or without active vitamin D analogs and calcimimetics were enrolled in the study. All participants had to sign an informed consent. The study was approved by the national regulatory authorities and was conducted in accordance with the principles of the International Council for Harmonization (ICH) and the Declaration of Helsinki (EudraCT Number 2013-000488-95). Within the NOPHOS study population, 632 participants had baseline and Week 12 samples available for measurement of iFGF23 and

proinflammatory cytokines [interferon (IFN)-γ, interleukin (IL)-1β, tumor necrosis factor (TNF) and IL-6]. All other parameters had been measured before.

Study protocol

The study protocol is described in Ketteler *et al.* [12].

Endpoints

For this *post hoc* analysis of the NOPHOS study, we analyzed the relationship between iFGF23 and cFGF23 and compared the potential of iFGF23 and cFGF23 to predict serum phosphate levels in hemodialysis patients. Furthermore, we explored the relationship of the proinflammatory cytokines IFN-γ, IL-1β, TNF and IL-6 as well as the iron parameters ferritin, transferrin, transferrin saturation and hemoglobin, with iFGF23 and cFGF23 in the hemodialysis population.

Biochemical assays

Blood sampling was done after a long dialysis-free interval (3 days) immediately before dialysis independent of daytime and were analyzed in a central laboratory for concentration of phosphate, calcium, intact PTH (iPTH), cFGF23, iron, transferrin, transferrin saturation, ferritin and C-reactive protein (CRP). T50 was measured by Calciscon AG. Plasma intact FGF23 at baseline and Week 12 was measured with the human iFGF23 ELISA (# 60-6600, Lot 173 373, Quidel Corporation) according to manufacturers' protocols. Unthawed plasma samples were aliquoted in two Eppendorf tubes to guarantee a smooth assay workflow. Standards and samples/internal assay controls were measured in duplicate and unicum, respectively. Samples were diluted at least 10-fold with assay diluent to be within the assay range. Samples which were still above the assay range were remeasured at higher dilutions. Inter-assay precision was monitored by measuring two internal assay controls in every plate ($n = 17$) which resulted for control sample I and II in an average of 40.3 $\pm$ 3.8 pg/mL and 431.6 $\pm$ 19.8 pg/mL with a coefficient of variance of 9.4% and 4.6%, respectively.

Cytokine measurements

Proinflammatory cytokines (IFN-γ, IL-1β, IL-6 and TNF) were measured in the plasma with the LEGENDplex Mix and Match system bead-based immunoassay (BioLegend) using a BD FACSCanto II flow cytometer (BD Bioscience) with a high throughput sampler according to the manufacture's protocol. All standards were prepared from the same Lot number (B299642). Standards and samples were measured in duplicate and individually, respectively. Standard curves and sample concentration were calculated with the LEGENDplex data analysis software Suite (version 2020.06.10). The appearance of bead populations (forward versus side scatter) and constant flow over time (forward scatter versus time) were checked in FlowJo Software version 10 (Becton, Dickinson and Company) (not shown).

Statistics

All statistical calculations and plots were carried out on Windows PCs applying the R-software (https://www.r-project.org/, version 3.6.3) and suitable software packages [34]. The data analysis was performed exploratory. Baseline characteristics were computed as mean $\pm$ standard deviation (SD) for metric variables and sample sizes (%) for qualitative variables, respectively. Biochemical variables were investigated with means, standard deviations, medians, interquartile ranges and compared between both treatment groups (placebo + PB and NAMR + PB, respectively) at baseline and after 12 weeks applying non-parametric Wilcoxon 2-sample tests. Correlations between biochemical variables were determined with Pearson's correlation coefficient. Data with skewed distributions were detected by means and medians, and related variance measures, and in this case the data were natural log-transformed prior to analysis. If possible, linear relationships were investigated applying simple linear regressions techniques. The recursive partitioning algorithm was applied in multivariable analyses to quantify the dependency structure of the present variable group [35, 36]. Finally, the effect of patient's characteristics, regulators of FGF23 expression as well as inflammatory and iron metabolism markers on the iFGF23 and cFGF23 variables were analyzed and estimated by means of linear mixed models with treatment and time as fixed and patient as random variable and presented in forest plots [maximum likelihood (ML) estimators and related 95% confidence intervals (CI)].

RESULTS

iFGF23, cytokine levels and iron parameters are similar between the NAMR + PB and placebo + PB group at 12 weeks of treatment

We analyzed 632 out of the 772 patients of the NOPHOS cohort of which baseline and Week 12 plasma samples were available for further biochemical analysis. This study population included 165 subjects of the placebo + PB group and 467 subjects of the NAMR + PB group. Baseline demographics and clinical characteristics of the NOPHOS subcohort were similar between treatment and placebo group (Table 1), as reported previously [12]. At baseline and Week 12 of treatment iFGF23, cFGF23 and cytokine levels showed skewed distributions. Therefore, all statistical analyses presented here were performed with these variables natural log transformed to generate more symmetric and approximately normal distributions at both time points (Supplementary data, Figs S1 and S2). At baseline, biochemical variables did not differ between the placebo + PB and NAMR + PB group (Table 2). As already described in Ketteler *et al.* [12], NAMR + PB treatment led to significantly lower serum phosphate and iPTH levels and increased serum calcium and T50 propensity at Week 12 compared with placebo + PB treatment (Table 2). Whereas iFGF23, cFGF23, IL-6, IL-1β, IFN-γ, TNF and all other parameters were similar in the placebo + PB and NAMR + PB group at Week 12.

NAMR + PB treatment reduced plasma iFGF23 and cFGF23 in a phosphate-dependent manner

Analyzing the change of log(iFGF23) levels from baseline to Week 12 with a linear mixed model revealed a significant greater reduction of log(iFGF23) in the NAMR + PB group as compared with placebo + PB group {maximum likelihood estimate (MLE) of a significant group difference [NAMR – placebo: –0.121 (95% CI –0.22, –0.03)]} (Table 3). Similarly, log(cFGF23) levels were reduced after 12 weeks in the NAMR + PB and placebo + PB group, respectively, but with much lower MLEs and no significant group difference [MLE of –0.059 (95% CI –0.17, 0.05)] (Table 3). Based on this analysis, iFGF23 is superior to cFGF23 in predicting the

Table 1: Baseline characteristics of placebo + PB and NAMR + PB treated patients [mean ± SD or samples size (%)].

Parameter	Placebo + PB ($n = 165$)	NAMR + PB ($n = 467$)
Age, years (mean ± SD)	62.2 ± 14.4	61.8 ± 13.6
Sex, *n* (%)		
Female	58 (35.2)	173 (37.0)
Male	107 (64.8)	294 (63.0)
Cause of CKD[a,b], *n* (%)	$n = 165$	$n = 467$
Nephrosclerosis/hypertension	43 (26.1)	114 (24.4)
Glomerulonephritis	33 (20.0)	119 (25.5)
Type 2 diabetes	30 (18.2)	99 (21.2)
Other	22 (13.3)	65 (13.9)
Polycystic kidney disease	22 (13.3)	55 (11.8)
Etiology unknown	18 (10.9)	21 (4.5)
Interstitial nephritis	14 (8.5)	25 (5.4)
Time from first RRT to screening, (mo.), median (IQR)	44 (57)	39 (56)
Dialysis mode, *n* (%)		
Hemodialysis	133 (80.6)	376 (80.5)
Hemodiafiltration	32 (19.4)	90 (19.3)
Hemofiltration	0 (0.0)	1 (0.2)
Time to dialysis mode, *n* (%)		
Morning	86 (52.1)	234 (50.1)
Midday/afternoon	52 (31.5)	141 (30.2)
Evening	22 (13.3)	79 (16.9)
Night	5 (3.0)	13 (2.8)
Kt/V (mean ± SD)	1.5 ± 0.3	1.5 ± 0.4
PB therapy, *n* (%)		
Monotherapy	133 (80.6)	366 (78.4)
Combination therapy	32 (19.4)	101 (21.6)
Calcium-containing PB, *n* (%)	85 (51.5)	244 (52.2)
Phosphate binder[a], *n* (%)		
Sevelamer	52 (31.5)	166 (35.5)
Calcium acetate and/or calcium carbonate	74 (44.8)	179 (38.3)
Calcium acetate and magnesium carbonate	23 (13.9)	100 (21.4)
Lanthanum carbonate	29 (17.6)	80 (17.1)
Aluminium	17 (10.3)	37 (7.9)
Colestilan	1 (0.6)	5 (1.1)
Sucroferric oxyhydroxide	1 (0.6)	1 (0.2)
Active vitamin D supplementation, *n* (%)	78 (47.3)	219 (46.9)
Calcimimetics, *n* (%)	38 (23.0)	93 (19.9)

[a]Multiple meanings possible.
[b]≥5% in either treatment group.
IQR: interquartile range; RRT: renal replacement therapy.

reduction in serum phosphate upon NAMR + PB or placebo + PB treatment. Adding phosphate as a covariate the treatment effect is no longer present, meaning the reduction in log(iFGF23) and log(cFGF23) by the treatment is phosphate dependent (data not shown).

iFGF23 and cFGF23 predict phosphate levels equally

Plasma iFGF23 and cFGF23 showed a significant strong positive correlation at baseline and Week 12 with Pearson correlation coefficients of 0.802 and 0.805, respectively (Fig. 1). Splitting the population equally with a bisector line it becomes obvious that lower log(iFGF23) levels have a tendency for higher log(cFGF23). Linear regression models of phosphate as dependent variable with iFGF23 or cFGF23, respectively, showed a similar coefficient of determination (R^2) meaning both, iFGF23 and cFGF23, have shown a similar capacity to predict serum phosphate in the current hemodialysis cohort (Fig. 2). In a multivariate model, the prediction capacity was evaluated by an alternative correlation analysis ("recursive partitioning") as described previously [35, 36]. The data at baseline and Week 12 was independently analyzed and all statistically important variables for the classification were determined (Supplementary data, Table S1). The statistical pattern recognition needed predefined classes to control the classification quality. The following four open and closed phosphate intervals were considered as meaningful cuts of the phosphate distribution to be used during classification: I_1 = (minimum, lower quartile Q_1), I_2 = (Q_1, median), I_3 = (median, Q_3) and I_4 = (Q_3, maximum). The analyses at baseline and Week 12 showed iFGF23 and cFGF23 together with T50 propensity as the most important predictors of phosphate levels whereas PTH played a minor role (Supplementary data, Tables S2–S5). Overall, the classification accuracy and kappa, a measure of the model reliability, were slightly higher for the Week 12 data. Independent of the time point, iFGF23 had a higher importance weight factor in contrast to cFGF23 (Supplementary data, Tables S2–S5) and slightly better recognition rates for phosphate values at the extremes (i.e. values <1st quartile or >3rd quartile) (Fig. 3a and b). The overall prediction accuracy of serum phosphate at Week 12 by the model with iFGF23 [56% (95% CI 52%, 60%)] or the model with cFGF23 [59% (95% CI 55%, 63%)] was similar. The other way around, phosphate was the variable with the highest impact in the prediction of iFGF23 and cFGF23 levels

Table 2: Biochemical variables at baseline and Week 12 of placebo + PB and NAMR + PB treated patients.

	Baseline			Week 12		
Parameter	Placebo + PB	NAMR + PB	P-value[a]	Placebo + PB	NAMR + PB	P-value[a]
Phosphate (mg/dL)	(*n* = 165)	(*n* = 467)		(*n* = 165)	(*n* = 473)	
Mean ± SD	6.03 ± 0.85	6.00 ± 0.91	.603	5.90 ± 1.35	5.36 ± 1.39	**<.0001**
Median (IQR)	5.90 (1.20)	5.90 (1.30)		5.80 (2.00)	5.20 (1.80)	
Calcium (mmol/L)	(*n* = 83)	(*n* = 249)		(*n* = 99)	(*n* = 308)	
Mean ± SD	2.13 ± 0.26	2.17 ± 0.21	.283	2.14 ± 0.22	2.22 ± 0.20	**<.0001**
Median (IQR)	2.16 (0.29)	2.19 (0.32)		2.16 (0.27)	2.23 (0.26)	
iPTH (pg/mL)	(*n* = 164)	(*n* = 467)		(*n* = 165)	(*n* = 473)	
Mean ± SD	311.4 ± 241.7	314.6 ± 292.4	.576	340.3 ± 306.7	292.3 ± 302.8	**.034**
Median (IQR)	246.0 (280.8)	240.0 (284.5)		258.0 (313.0)	225.0 (240.0)	
cFGF23 (RU/mL)	(*n* = 159)	(*n* = 449)		(*n* = 164)	(*n* = 443)	
Mean ± SD	5467 ± 6666	5205 ± 7097	.531	6474 ± 10 184	5143 ± 8611	.254
Median (IQR)	2827 (5816)	2764 (4580)		2601 (5377)	2231 (4803)	
iFGF23 (pg/mL)	(*n* = 165)	(*n* = 466)		(*n* = 165)	(*n* = 473)	
Mean ± SD	8944 ± 15 042	8977 ± 20 002	.58	8319 ± 13 445	9481 ± 25 049	.354
Median (IQR)	3405 (9407)	3083 (7367)		2622 (7935)	2215 (7373)	
T50 (min)	(*n* = 165)	(*n* = 456)		(*n* = 164)	(*n* = 457)	
Mean ± SD	155.7 ± 85.0	151.1 ± 76.0	.827	157.4 ± 84.6	174.5 ± 89.2	**.021**
Median (IQR)	124.0 (109.0)	127.5 (96.5)		129.5 (106.8)	153.0 (134.0)	
Transferrin (g/dL)	(*n* = 165)	(*n* = 467)		(*n* = 165)	(*n* = 473)	
Mean ± SD	1.81 ± 0.35	1.80 ± 0.35	.587	1.80 ± 0.38	1.77 ± 0.35	.384
Median (IQR)	1.78 (0.41)	1.77 (0.42)		1.77 (0.42)	1.75 (0.41)	
TSAT (%)	(*n* = 165)	(*n* = 467)		(*n* = 165)	(*n* = 473)	
Mean ± SD	27.7 ± 13.9	26.9 ± 13.5	.689	25.9 ± 14.0	25.7 ± 13.3	.736
Median (IQR)	23.9 (14.3)	24.6 (15.0)		21.7 (12.6)	22.7 (12.0)	
Ferritin (µg/L)	(*n* = 165)	(*n* = 467)		(*n* = 165)	(*n* = 473)	
Mean ± SD	742.6 ± 583.0	696.6 ± 555.6	.305	715.6 ± 591.2	829.2 ± 657.6	.053
Median (IQR)	620.0 (694.0)	544.0 (642.5)		574.0 (709.0)	698.0 (789.0)	
CRP (mg/L)	(*n* = 165)	(*n* = 467)		(*n* = 165)	(*n* = 473)	
Mean ± SD	10.9 ± 18.4	10.5 ± 15.7	.433	10.8 ± 15.5	16.6 ± 30.6	.110
Median (IQR)	4.8 (9.2)	5.3 (10.6)		4.9 (8.8)	6.1 (14.3)	
IL-6 (pg/mL)	(*n* = 160)	(*n* = 450)		(*n* = 159)	(*n* = 451)	
Mean ± SD	33.6 ± 36.6	43.4 ± 98.6	.348	36.0 ± 39.9	47.0 ± 74.2	.585
Median (IQR)	23.2 (27.2)	23.9 (31.3)		24.1 (24.1)	23.9 (35.6)	
IL-1ß (pg/mL)	(*n* = 83)	(*n* = 226)		(*n* = 73)	(*n* = 215)	
Mean ± SD	8.3 ± 12.8	18.4 ± 137.9	.404	7.9 ± 8.6	17.8 ± 99.5	.547
Median (IQR)	3.8 (7.6)	4.2 (7.1)		5.4 (6.5)	3.9 (6.6)	
IFN-γ (pg/mL)	(*n* = 119)	(*n* = 328)		(*n* = 115)	(*n* = 368)	
Mean ± SD	5.2 ± 7.1	13.8 ± 144.8	.353	5.0 ± 6.9	12.2 ± 104.7	.907
Median (IQR)	3.0 (4.4)	2.5 (4.0)		2.9 (4.3)	3.0 (4.5)	
TNF (pg/mL)	(*n* = 77)	(*n* = 225)		(*n* = 74)	(*n* = 227)	
Mean ± SD	27.1 ± 45.7	77.6 ± 686.3	.473	32.9 ± 43.5	69.7 ± 491.5	.136
Median (IQR)	13.8 (26.3)	12.6 (24.5)		19.8 (33.7)	12.8 (21.3)	

[a]Wilcoxon 2-sample test (*P*-values ≤0.05 are printed in bold).
IQR: interquartile range; TSAT: transferrin saturation.

with importance weight factors that were 35% and 45% higher for iFGF23 compared with cFGF23 at baseline and Week 12, respectively (Supplementary data, Tables S6–S9). Again, the classification accuracy and kappa were higher for the Week 12 data compared with baseline. At Week 12, iFGF23 had a higher overall classification accuracy than cFGF23 [56% (95% CI 52%, 60%) versus 50% (95% CI 46%, 54%)] including higher classification rates for iFGF23 in all four classes. However, this trend was not confirmed in the baseline data (Fig. 3c and d).

Predictors of iFGF23 and cFGF23 in hemodialysis patients

Patient characteristics, regulators of FGF23 expression as well as markers of inflammation and iron metabolism were analyzed by linear mixed models with iFGF23 or cFGF23 as dependent, treatment and time as fixed, and patient as random variables. The results are visualized in a forest plot in Fig. 4.

Sex, age and body mass index

iFGF23 levels were estimated as higher in men than women, however, the CI was wide [MLE 0.339 (95% CI 0.07, 0.61)]. Yet, sex does not estimate cFGF23 in this cohort of hemodialysis patients. Age was predictive for both iFGF23 and cFGF23, meaning with older aged patients have lower iFGF23 [MLE –0.025 (95% CI –0.034, –0.016)] and cFGF23 [MLE –0.011 (95% CI –0.018, –0.005)], whereas body mass index (BMI) was not predictive of either iFGF23 nor cFGF23.

Table 3: Changes of plasma iFGF23 and cFGF23 from baseline to Week 12.

Variable	Statistic[a]	NAMR + PB	Placebo + PB	P-value	Test
log(iFGF23)	*n*	456	163		
	Mean (SD)	–0.245 (0.798)	–0.130 (0.704)	.0112	Linear mixed model *t*-test
	Median (IQR)	–0.214 (0.909)	–0.153 (0.747)	.0867	
		Point estimates (NAMR + PB—placebo + PB) and 95% CI			
	Mean estimate	–0.115, 95% CI (–0.25, +0.02)			
	ML estimate	–0.121, 95% CI (–0.22, –0.03)			
log(cFGF23)	*N*	424	157		
	Mean (SD)	–0.085 (0.843)	–0.051 (0.948)	.2892	Linear mixed model *t*-test
	Median (IQR)	–0.099 (0.752)	–0.037 (0.680)	.6802	
		Point estimates (NAMR + PB—placebo + PB) and 95% CI			
	Mean estimate	–0.034, 95% CI (–0.20, +0.13)			
	ML estimate	–0.059, 95% CI (–0.17, +0.05)			

[a]*n* = number of evaluable data records.
IQR: interquartile range.

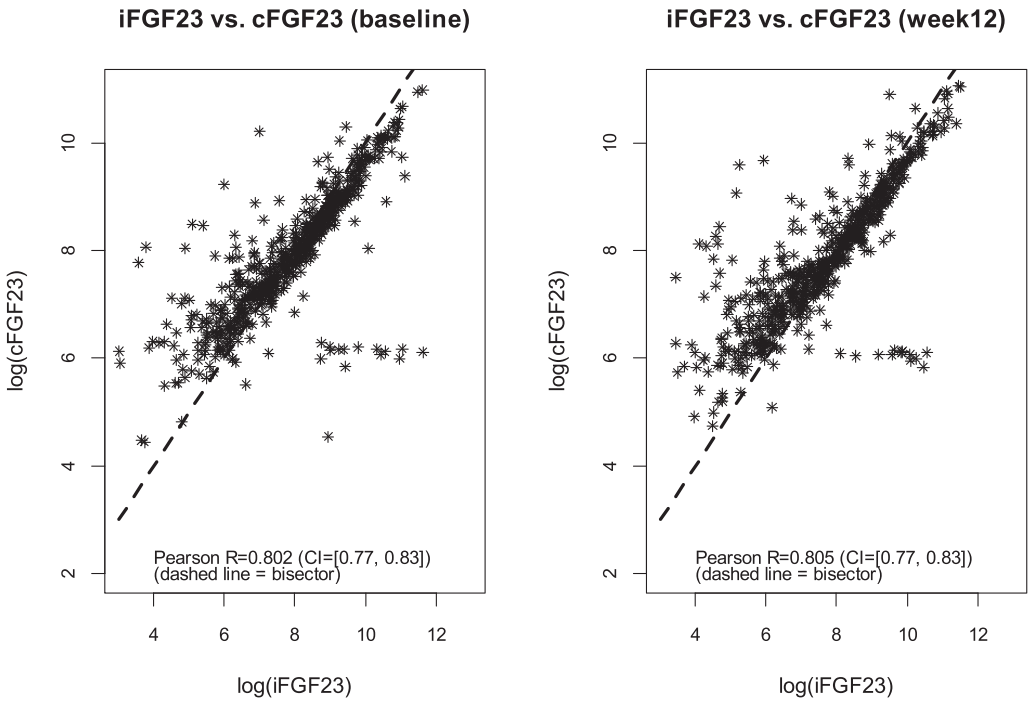

Figure 1: Correlation between log(iFGF23) and log(cFGF23) at baseline and Week 12 in hemodialysis patients with placebo + PB or NAMR + PB treatment. The dashed line represents the bisector line.

Regulators of FGF23 expression

As expected, both, higher serum phosphate and iPTH levels were highly predictive for higher iFGF23 and cFGF23 levels, respectively, with very narrow CIs. The change in iFGF23 and cFGF23 per unit change of the predictor variable is higher in phosphate compared with iPTH.

Markers of inflammation

Neither of the cytokines measured nor CRP were important predictors of plasma iFGF23 in hemodialysis patients. CRP and IL-6 were predictors of cFGF23, however the size of MLE and the wide CI makes it clinically irrelevant [MLE = 0.005 (95% CI 0.003, 0.008), MLE = 0.0016 (95% CI 0.0009, 0.002)].

Markers of iron metabolism

Ferritin, transferrin and transferrin saturation were no predictors for plasma iFGF23 and cFGF23. Hemoglobin was a predictor of iFGF23 but not cFGF23, however the size of MLE and the very wide CI makes it clinically irrelevant [0.087 (95% CI 0.05, 0.13)].

DISCUSSION

Hyperphosphatemia is a challenging problem in patients with ESKF and its treatment is inevitable to prevent the associated

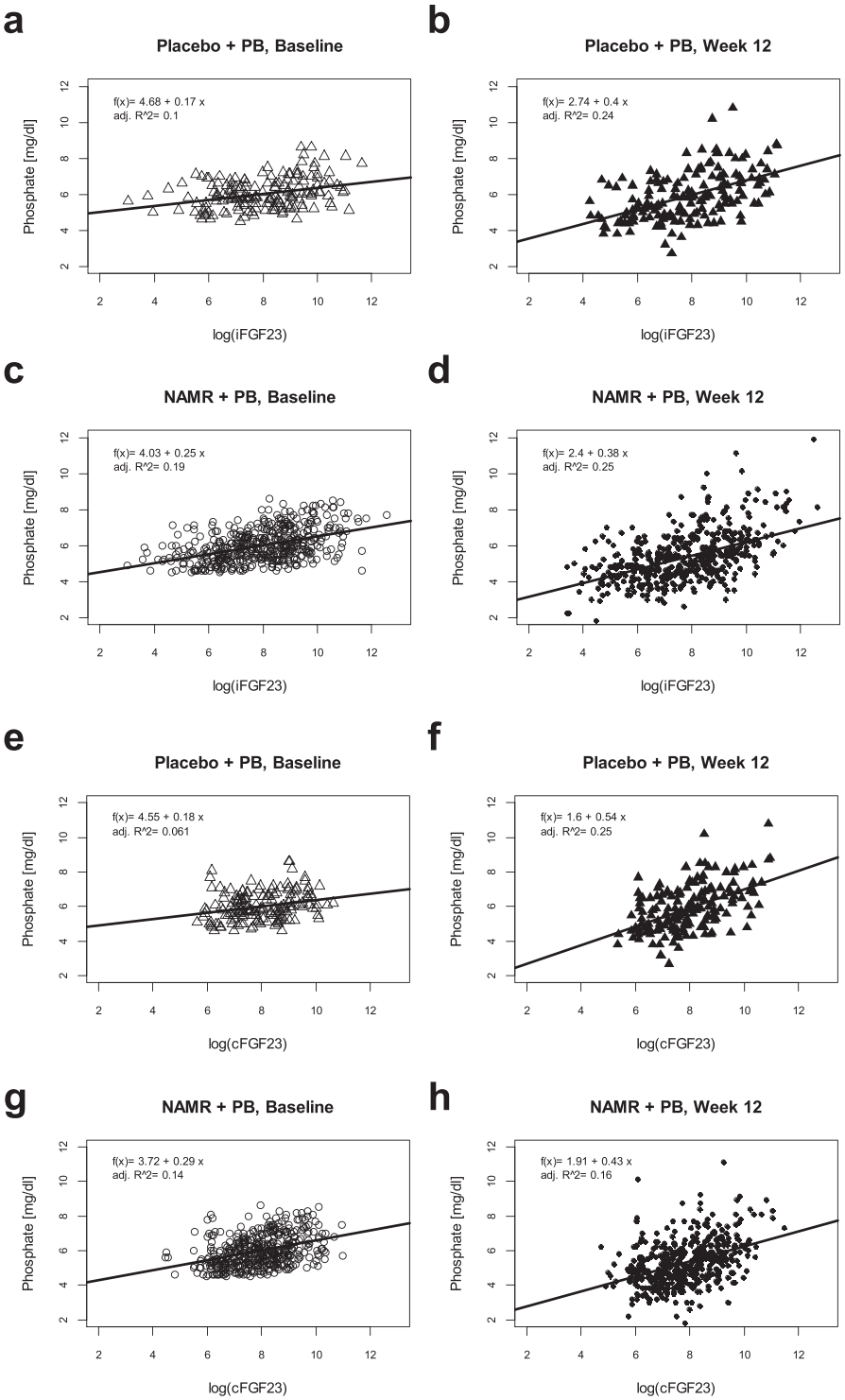

Figure 2: Linear regression model with phosphate as dependent variable and either iFGF23 (**a–d**) or cFGF23 (**e–h**) at baseline (a, c, e, g) or Week 12 (b, d, f, h) in hemodialysis patients with placebo + PB (a, b, e, f) or NAMR + PB (c, d, g, h) treatment.

risk of cardiovascular disease. The suitability of the phosphaturic hormone FGF23 as a marker of kidney function and exposure to phosphate is controversially discussed not least because of the many factors known to regulate FGF23 levels and limited data providing evidence for the suitability of iFGF23 or cFGF23 as appropriate surrogate marker in the population at risk. In the NOPHOS cohort of hemodialysis patients with hyperphosphatemia, we found first a very strong correlation between iFGF23 and cFGF23, second iFGF23 and cFGF23 together with T50 propensity manifested as strong predictors of serum phosphate, and third phosphate and PTH appeared as the strongest predictors of iFGF23 and cFGF23 while markers of inflammation and iron metabolism had a minor impact on the prediction of iFGF23 and cFGF23.

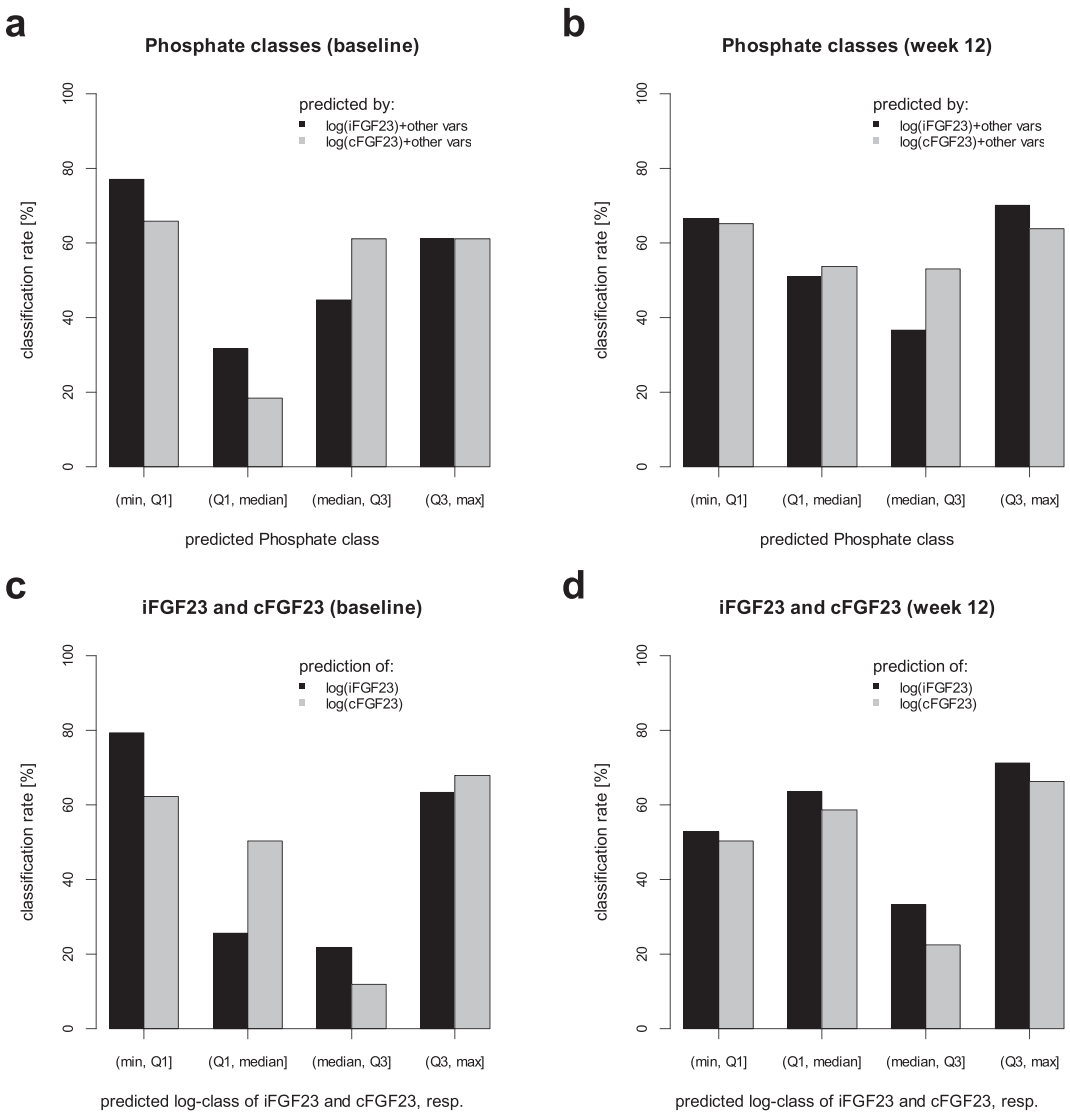

Figure 3: Classification rates of phosphate classes (**a**, **b**) and log(iFGF23) or log(cFGF23) classes (**c**, **d**), respectively, at baseline (a, c) and Week 12 (b, d) determined by recursive partitioning in hemodialysis patients with placebo + PB or NAMR + PB treatment.

In cohorts with peritoneal and hemodialysis patients, iFGF23 and cFGF23 are tightly correlating with each other [37, 38], however this correlation is weaker in healthy volunteers [39]. The use of different units in human iFGF23 and cFGF23 ELISA assays makes it difficult to get an impactful proportion of iFGF23 per cFGF23. Determined by western blot, it was observed that dialysis patients have higher proportion of iFGF23 compared with pre-dialysis patients and healthy volunteers [17]. Furthermore, FGF23 in serum of ESKF patients is biologically active as demonstrated in a cell-based reporter assay [37]. Mice fed a high phosphate diet for 2 weeks have higher plasma iFGF23, however not *Fgf23* mRNA expression but rather *Galnt3* mRNA expression is increased in bone, a gene encoding the enzyme polypeptide N-acetylgalactosaminyltransferase 3 which is responsible for O-linked glycosylation of FGF23 [40]. O-linked glycosylation prevents FGF23 from rapid cleavage, prolonging its half-life time [41]. Upregulation of *Galnt3* mRNA expression by high phosphate diet would imply a higher percentage of iFGF23 in the setting of hyperphosphatemia [40]. In the NOPHOS cohort, we observed a tight correlation between iFGF23 and cFGF23, however with a trend of lower iFGF23 having higher cFGF23 levels. This suggests that ESKF patients with hyperphosphatemia have a high percentage of iFGF23 which might be achieved by different means such as increased O-glycosylation, constant cleavage despite increased FGF23 production, reduced cleavage with constant FGF23 production or a combination of these. The lack of correlation in healthy volunteers suggests that under conditions of normal kidney function and absence of hyperphosphatemia other factors add to the determination of the proportion of iFGF23 and cFGF23 [39].

Phosphate is the main driver of plasma FGF23 in ESKF and hemodialysis patients with reoccurring hyperphosphatemia after phosphate binder withdrawal having a concomitant increase in plasma iFGF23 [14, 42]. Here we showed that in hemodialysis patients with hyperphosphatemia both iFGF23 and cFGF23 similarly predict serum phosphate levels independently of time point and treatment; however, only iFGF23 is sensitive enough to predict the decrease of serum phosphate observed in

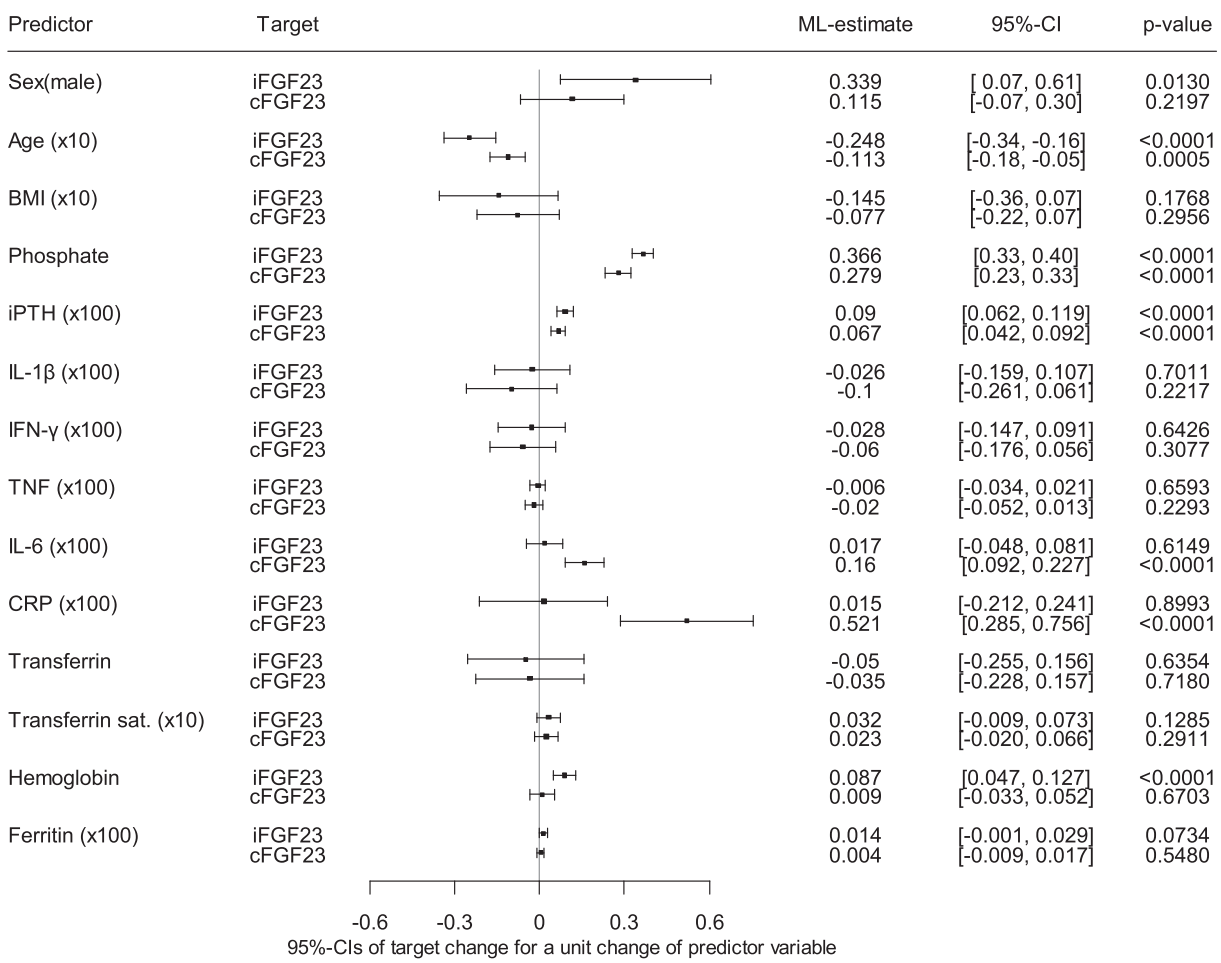

Figure 4: ML estimate and 95% Cis of predictors of plasma iFGF23 and cFGF23 analyzed by linear mixed model in the current cohort of hemodialysis patients with placebo + PB or NAMR + PB treatment. To visualize all predictors in a single forest plot, age, BMI, transferrin saturation, iPTH, IL-1β, IFN-γ, TNF, IL-6, CRP and ferritin were multiplied by 10 or 100, respectively.

patients treated with NAMR + PB compared with placebo + PB from baseline to Week 12. Similarly in hemodialysis patients with hyperphosphatemia treated with tenapanor, the change in serum phosphate correlated positively with changes in plasma iFGF23 [42]. This was also observed in hemodialysis patients with hyperphosphatemia treated either with the PB sucroferric oxyhydroxide or sevelamer, however the correlation was weaker [43]. By alternative correlation analysis ("recursive partitioning") we confirmed the importance of iFGF23 and cFGF23 together with T50 in predicting serum phosphate.

Taken together, iFGF23 and cFGF23 showed similar capability to predict serum phosphate, however iFGF23 was more precisely predicting the decrease in serum Pi caused by NAMR + PB treatment. This could be due to the antagonistic action of the C-terminal FGF23 fragment on FGF23-induced phosphaturia [44]. Therefore, in hemodialysis patients with hyperphosphatemia measuring iFGF23 might be superior when using it as marker of exposure to phosphate [16].

Plasma FGF23 is regulated by calcitriol and PTH, two factors forming together with FGF23 a hormonal network to regulate phosphate homeostasis. Calcitriol induces FGF23 synthesis in bone and increases intestinal phosphate absorption [14, 45, 46]. The modulation of FGF23 by calcitriol was not investigated as calcitriol was not measured in this cohort and a portion of patients received calcitriol supplements. PTH stimulates FGF23 expression in bone via the transcription factor Nurr1 [47]. We found that in the NOPHOS cohort, phosphate and iPTH were highly predictive for plasma iFGF23 and cFGF23, as described previously [48]. However, performing alternative correlation analysis by recursive partitioning, serum phosphate stands out as the predictor with the largest weight in the model independent of the timepoint and treatment.

The association between FGF23 and age or sex is controversially discussed in the literature [24, 49–51]. In the NOPHOS cohort, older patients have lower iFGF23 and cFGF23. However, considering the study population it might underlie a survival bias, as older hemodialysis patients with higher plasma FGF23 are more likely to die prematurely.

Elevated plasma FGF23 levels have been associated with all cause and cardiovascular morbidity and mortality in CKD patients as well as in the general population [38, 49]. The underling mechanism for this association is unclear but might include its potential involvement in inflammatory processes or anemia [52–54]. Proinflammatory stimuli and bacterial pathogens stimulate FGF23 expression [18, 20, 52]. Furthermore, in CKD and non-CKD populations, proinflammatory stimuli have been

associated with elevated plasma FGF23 [20, 55–57]. In contrast, we did not find IL-1β, IFN-γ or TNF as a predictor of iFGF23 or cFGF23. Whereas IL-6 and CRP were predictive for cFGF23 but not iFGF23, however the interquartile range was wide for both. Iron deficiency increases FGF23 production in bone and increases FGF23 cleavage resulting in high plasma cFGF23 but normal iFGF23 levels [22]. In ESKF, anemia is prevalent in over 50% of the patients [58] and FGF23 cleavage is either decreased [17] or the cleavage machinery is unchanged, and the higher production rate of FGF23 results in a higher percentage of iFGF23. Therefore, anemia could contribute to the extremely high levels of iFGF23 seen in ESKF patients [59]. Indeed, treatment of patients undergoing dialysis with ferric citrate hydrate PB not only reduced plasma iFGF23 but also increased serum iron and ferritin and reduced the dose of erythropoietin and iron supplementation compared with the lanthanum carbonate control group [60]. However, looking at the associations between parameters of iron metabolism and FGF23 in clinical studies the data are inconclusive [27–33]. In the NOPHOS cohort, neither transferrin, transferrin saturation nor ferritin were predictors for iFGF23 and cFGF23. Hemoglobin was a predictor of plasma iFGF23, but not cFGF23. We suggest that in dialysis patients with hyperphosphatemia the relationship between inflammation and FGF23 as well as anemia and FGF23 is masked by the urgent need to control serum phosphate.

In summary, we found a strong correlation between iFGF23 and cFGF23 and that iFGF23 and cFGF23 together with T50 propensity manifested as very strong predictors of serum phosphate. Furthermore, serum phosphate and iPTH appeared as the strongest predictors of iFGF23 and cFGF23 while markers of inflammation and iron metabolism had a minor impact in the prediction of iFGF23 and cFGF23 in dialysis patient with hyperphosphatemia. Hence, we conclude that lowering serum phosphate in ESKF patients depends highly on iFGF23, which is correlated to plasma cFGF23. Based on the capability of serum phosphate to predict plasma FGF23, our data are consistent with the important role of FGF23 in the regulation of serum phosphate levels above other regulatory mechanism in ESKF.

SUPPLEMENTARY DATA

Supplementary data are available at *ckj* online.

AUTHORS' CONTRIBUTIONS

D.E.-S., I.R.-A. and C.A.W designed the present study. M.K., A.W., A.R.R., B.H., M.K. and R.A. designed the NOPHOS study cohort. A.K.H., E.M.P.A. and D.E.-S. conducted experiments. H.L. performed statistical data analysis. D.E.-S., I.R.-A., C.A.W., B.H. and M.K. interpreted the results. D.E-S. and I.R.-A. wrote the manuscript. C.A.W. and Medice AG raised funding. All authors read the manuscript, contributed to editing and approved the final version.

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DATA AVAILABILITY STATEMENT

Due to the nature of this research, participants of this study did not agree for their data to be shared publicly, therefore supporting data are not available. MEDICE will share the data upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors declare that they are not aware of any conflicts of interest and that the results presented in this paper have not been published previously in whole or part, except in abstract format. C.A.W. reports honoraria from Medice, Advicenne, Kyowa Kirin and Chugai. A.P. is an employee of Calciscon AG and reports holding stock in Calciscon AG, which commercializes the T50 test.

(See related article by Magagnoli et al. The open system of FGF-23 at the crossroad between additional P-lowering therapy, anemia and inflammation: how to deal with the intact and the C-terminal assays? *Clin Kidney J* (2023) 16: 1543–1549.)

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