Aus der Klinik für Nephrologie und Intensivmedizin
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Non-oxidized PTH - but not oxidized PTH - stimulates FGF23 and increases only moderately in CKD patients

Im Gegensatz zu oxidiertem PTH zeigt nicht oxidiertes PTH nur einen moderaten Anstieg in CKD Patienten und ist in der Lage Fgf23 zu stimulieren

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Shufei Zeng

aus Guangdong, China

Datum der Promotion:03.12.2021

TABLE OF CONTENT

List of Abbreviations

FGF23	Fibroblast growth factor 23
cFGF23	C-terminal FGF23
iFGF23	Intact FGF23
CKD	Chronic kidney disease
PTH	Parathyroid hormone
iPTH	Intact PTH
n-oxPTH	Non-oxidized PTH
oxPTH	Oxidized PTH
Met8(ox)-PTH	PTH oxidized at Met8
Met8(ox)-PTH	PTH oxidized at Met18
Met8, Met18(di-ox)-PTH	PTH oxidized at Met 8 and Met18
GFR	Glomerular filtration rate
eGFR	Estimated glomerular filtration rate
GPCR	G-protein-coupled receptor
Nurr1	Nuclear receptor-associated protein-1
HF	Heart failure
BAP	Bone alkaline phosphatase
HbA1c	Hemoglobin A1c
TBP	tatabox-binding protein
4C	Cardiovascular Comorbidity in Children with Chronic Kidney Disease
PTHR1	Parathyroid hormone/parathyroid hormone-related protein receptor 1

1. Abstrat

1.1 Deutsch

Einleitung: Das Nebenschilddrüsenhormon (PTH) und der Fibroblasten-Wachstumsfaktor 23 (FGF23) sind Regulatoren des Vitamin-D-Stoffwechsels und der renalen Phosphatausscheidung. Bei chronischer Nierenerkrankung (CKD) steigen die Konzentrationen von zirkulierendem FGF23 und PTH mit abnehmender Nierenfunktion progressiv an. Die Oxidation von PTH an 2 Methioninresten (Positionen 8 und 18) verursacht einen Verlust der biologischen Funktion. Der Einfluss von nicht-oxidiertem PTH (n-oxPTH) und oxidiertem PTH (oxPTH) auf die FGF23-Synthese und wie die n-oxPTH- und oxPTH-Konzentrationen durch CKD beeinflusst werden, ist jedoch noch unbekannt.

Methoden: Wir analysierten die Auswirkungen von PTH1-34 und seinen oxidativen Derivaten auf die FGF23-Genexpression in osteoblastenähnlichen UMR106-Zellen. Darüber hinaus wurde die Beziehung zwischen n-oxPTH bzw. oxPTH mit FGF23 bei 620 Kindern mit CKD untersucht.

Ergebnisse: N-oxPTH stimulierte die FGF23-mRNA-Synthese in vitro, während die Oxidation von PTH insbesondere an Met8 zu einer deutlich schwächeren Stimulation von FGF23 führte. Wenn beide Methioninreste (Positionen 8 und 18) oxidiert wurden, war der Effekt noch stärker. In der klinischen Studie ist n-oxPTH, aber nicht OxPTH, positiv und unabhängig von bekannten Confoundern mit FGF23 assoziiert. Mit verschlechterneder Nierenfunktion stiegen zudem intaktes PTH (iPTH) und oxPTH signifikant an, während das biologisch aktieve n-oxPTH nur mäßig zunahm.

Fazit: N-oxPTH, aber nicht oxPTH, stimuliert die FGF23-Genexpression. Der Anstieg von PTH bei abnehmender GFR ist hauptsächlich auf eine Erhöhung von oxPTH in fortgeschritteneren Stadien der CKD zurückzuführen.

1.2 English

Introduction: Parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) are regulators of vitamin D metabolism and renal phosphate excretion. **I**n chronic kidney disease (CKD), the concentrations of circulating FGF23 and PTH increase progressively with decreasing renal function. The oxidation of PTH at 2 methionine residues (positions 8 and 18) causes a loss of biological function. However, the influence of non-oxidized PTH (n-oxPTH) and oxidized PTH (oxPTH) on FGF23 synthesis and how n-oxPTH and oxPTH concentrations are affected by CKD is still unknown.

Methods: We analyzed the effects of PTH1-34 and its oxidative derivatives on FGF23 gene expression in osteoblast-like UMR106 cells. Furthermore, we investigated the relationship between n-oxPTH and oxPTH with FGF23 in 620 children with CKD.

Results: N-oxPTH stimulated the FGF23 mRNA synthesis in vitro, whereas the oxidation of PTH especially at Met8 led to a significantly weaker stimulation of FGF23. When both methionine residues (positions 8 and 18) were oxidized, the effect was even stronger. In the clinical study, n-oxPTH, but not oxPTH, is positively and independently of known confounders associated with FGF23. In addition, as renal function deteriorated, intact PTH (iPTH) and oxPTH increased significantly, while n-oxPTH increased only moderately.

Conclusion: Non-oxPTH, but not oxPTH, stimulates FGF23 gene expression. The rise in PTH with a decrease in GFR is mainly due to an elevation of oxPTH in more advanced stages of CKD.

2. Introduction

Fibroblast growth factor 23 (FGF23), a 32 kDa glycoprotein, is mainly produced by osteoblasts and osteocytes in bone under physiological conditions.(1) It plays a central role in the regulation of phosphate homeostasis by the coordinated modulation of kidney phosphate handling, parathyroid hormone (PTH) secretion and vitamin D metabolism.(2) In the kidney, FGF23 controls reabsorption of phosphate by inducing the internalization of sodium–phosphate cotransporters (NaPi-IIa and NaPi-IIc) from the luminal side into proximal nephron segments. This reduction in the membrane expression of NaPi transporters restricts the kidney's phosphate resorption capacity and stimulates phosphate excretion.(2) FGF23 also downregulates the 1α-hydroxylase enzyme to inhibit the activation of 25-hydroxycholecalciferol into 1,25-dihydroxycholecalciferol and upregulates 24-hydroxylase to promote vitamin D catabolism. The resulting decrease in circulating active vitamin D levels limits gastrointestinal absorption of phosphate, which is an integral component of the phosphate lowering effects of FGF23.(2) Moreover, the secretion of PTH in the parathyroid gland is also under the control of FGF23 and PTH releases phosphate from the bone, so this is another integral part of the phosphate-lowering action of FGF23.(2)

In chronic kidney disease (CKD), with a progressive decrease in glomerular filtration rate (GFR), an increase in FGF23 plasma concentration occurs very early in the disease, even before the appearance of significant hyperparathyroidism (measured by the currently used intact PTH assays) and hyperphosphatemia. (3, 4) Whether elevated FGF23 levels measured at a single time or slowly increasing FGF23 trajectories by serial measurements are strongly associated with an increased risk of mortality in patients with CKD. (5)

Known regulators of FGF23 production include PTH(6), $1,25(OH)_2D_3$(7), inflammation(8), the iron status(8), dietary phosphate(9), calcium(10), or TGFβ2(11). Most previous studies have shown a very close correlation between PTH (measured by conventional intact PTH assays) and FGF23 serum levels. It is believed that PTH and FGF23 mutually regulate each other in a negative feedback loop in which FGF23

suppresses PTH synthesis and PTH in turn stimulates FGF23 production.(6) The prevailing assumption is that the binding of FGF23 to FGFR1c and αKlotho, which are co- expressed on parathyroid cells, mediated the suppression of PTH by FGF23.(2) It is noteworthy that concentrations of both PTH and FGF23 are high in CKD, as downregulation of the FGF23 receptor complex Klotho-FGFR1 leads to a resistance of the parathyroid to FGF23.(2) Secondary hyperparathyroidism (SHP) is the main factor behind the high levels of FGF23 in CKD.(6) In vivo, parathyroidectomy both prevented and corrected elevated FGF23 serum levels in rats with short-term adenine-induced kidney failure.(6) In addition, in vitro PTH elevated FGF23 mRNA levels in osteoblast-like UMR106 cells of the rat via both the PKA and Wnt pathways.(6) Later, Meir et al.(12) demonstrated that the effect of PTH to increase FGF23 transcription is mediated by the nuclear receptor-associated protein-1 (Nurr1) in vitro and in vivo.

The PTH molecule, a 84-amino acids containing peptide hormone, is synthesized and secreted by the main cells of the parathyroid gland. There are two methionine amino acids at positions 8 and 18, which can be oxidized in vivo, either individually or together (13-15). (Figure 1A) The receptor binding site is also located within this region of the PTH molecule. Their oxidation can lead to reduced binding to the PTH receptor. A number of studies have clearly shown that the PTH oxidation is critical for the biologic activity of PTH. In our previous study, we have described the presence of a varying level of oxidized PTH (oxPTH) in the circulation of patients after kidney transplantation, during dialysis and with CKD.(13) The relationship between non-oxidized PTH (n-oxPTH) and oxPTH is highly variable and cannot currently be predicted in individual patients.(13-15)

So far, three generations of PTH assays have been developed.(16) The second and third generation of PTH assays are currently in use and their clinical performance appears to be comparable. The first generation of PTH assays—RIAs—have major limitations and is no longer used. As it is a typical single antibody directed against the middle region of the PTH1-84 sequence and detects a mixture of mature PTH1-84, N-terminal PTH fragments and bio inactive C-terminal fragments. (Figure 1B) Since the biological activity of the hormone was discovered in the amino-terminal residues of the molecule and

truncated PTH fragments exist, especially in patients with CKD, leading to falsely elevated results in the assay, second-generation assays have been developed that allegedly measure "intact PTH". The second generation of PTH assays is immunoassays at two sites using a pair of affinity-purified antibodies specific for two different regions of the PTH molecule. The capture antibody was directed against the C-terminal region of the hormone (amino acids 26-32 or 39-84), while the detection antibody was specific for the N-terminal (normally against amino acids 12-24). (Figure 1B) This type of assay design increased the specification of the PTH assays by avoiding cross-reactivity with C-terminal PTH fragments. Next, the third generation of PTH assays was developed. The difference to the second generation PTH assays is that the detection antibody epitope has been further directed towards the N-terminus of PTH1-84 towards amino acids 1-4. (Figure 1B) There is no doubt that the development of second and third generation of PTH assays was a huge step towards the goal of developing PTH assays that measure bioactive PTH only. However, they still have major shortcomings, ignoring a second biological process that changed native PTH1-84 (unlike PTH fragmentation): the PTH oxidation. A new assay system was therefore developed to measure only n-oxPTH. The detection process for n-oxPTH is described in the methods section.

In the current study, we explored the relationship of oxidized and non-oxidized PTH with FGF23. We analyzed the effects of PTH1-34 and its oxidative derivatives on FGF23 gene expression in UMR106 osteoblast-like cells. In addition, we investigated the relationship between oxPTH, n-oxPTH, and iPTH (the sum of oxPTH and n-oxPTH) with FGF23 in 620 children with CKD, considering known confounders(6, 9, 10, 17), and also analyzed the relationship between GFR and n-oxPTH, oxPTH, iPTH, as well as FGF23.

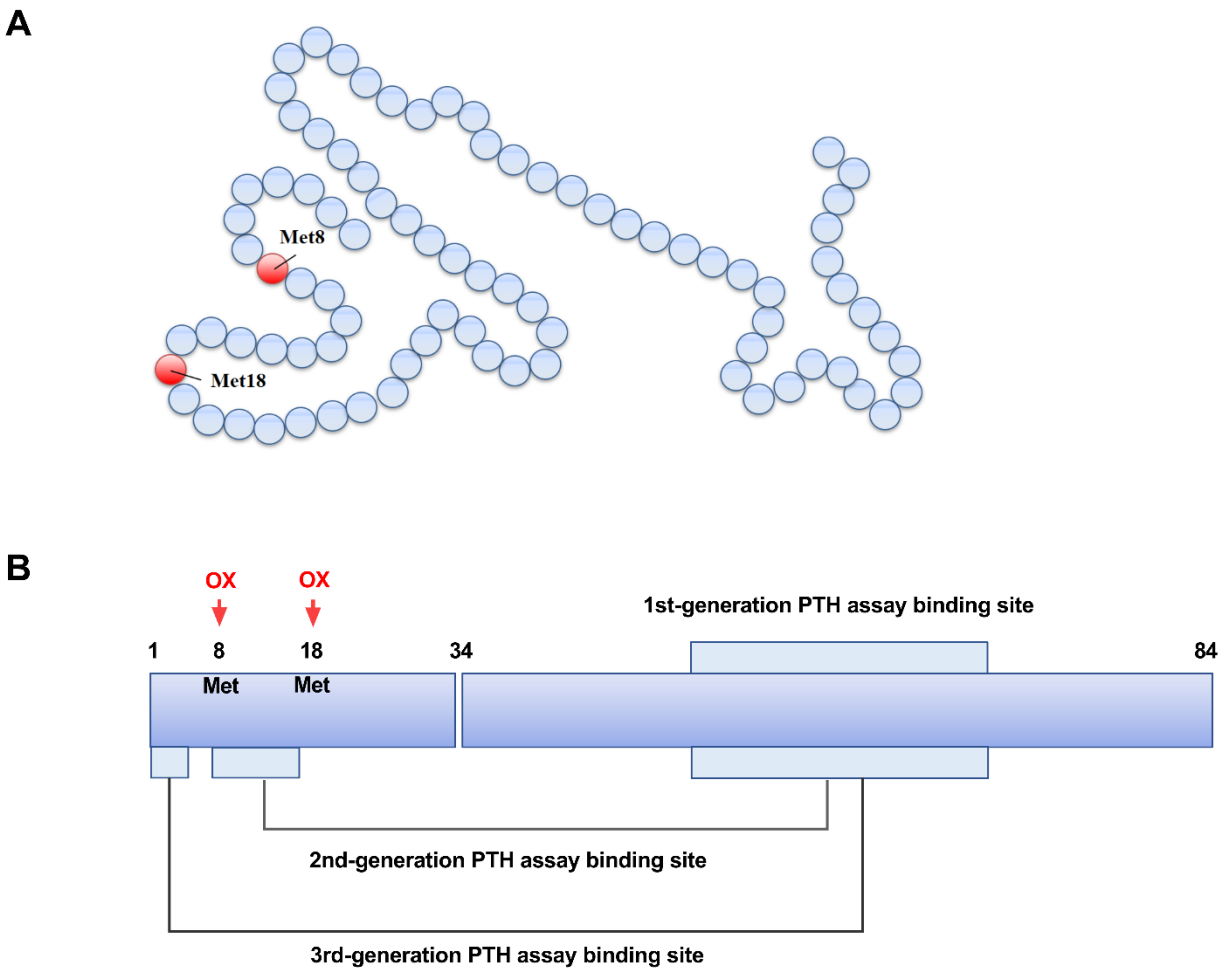


Figure 1. Schematic diagram of the full-length PTH (1–84) molecule and first to third generation parathyroid hormone (PTH) assays target different regions of PTH molecule.

(A) There are two methionine amino acids at positions 8 and 18 of PTH 1–84, which can be oxidized in vivo, either individually or together. Their oxidation can lead to reduced binding to the PTH receptor.

(B) The first generation of PTH assays (RIAs) is a typical single antibody directed against the middle region of the PTH1-84 sequence. The second generation of PTH assays is immunoassays at two sites using a pair of affinity-purified antibodies specific for two different regions of the PTH molecule. The capture antibody was directed against the C-terminal region of the hormone (amino acids 26-32 or 39-84), while the detection antibody was specific for the N-terminal (normally against amino acids 12-24). The difference of the third generation of PTH assays to the second generation PTH assays is that the detection antibody epitope has been further directed towards the N-terminus of PTH1-84 towards amino acids 1-4. Methionine residues that might be oxidized are marked with red triangles.

This figure is adapted from our previous publication.(16)

3. Materials and methods

3.1 Patients

We analyzed plasma samples from a prospective multi-center observational cohort study, in 6 to 17-year-old children participating in the Cardiovascular Comorbidity in Children with Chronic Kidney Disease (4C) Study, with a GFR of 10 to 60 ml/min/1.73 m² at baseline. (18, 19) The study protocol was approved by the ethics committee of the University of Heidelberg. Patients´ custodians gave their informed consent, and patient information and consent forms (translated into the national language) were checked and approved by the local Institutional Review Boards in each participating institution. Children's demographic data (age, gender, BMI, height etc.) were obtained from the patients' medical records. A previously published GFR estimating formula for children with CKD was used to calculate the estimated glomerular filtration rate (eGFR): GFR(ml/min/1.73m^2)=39.1[height(m)/Scr(mg/dl)](0.516)x[1.8/cystatin C (mg/L)](0.294)[30/BUN (mg/dl)](0.169)[1.099](male)[height (m)/1.4](0.188).(20) At the beginning of the study and every 6 months during follow-up period, blood and urine samples were collected, stored and analyzed in a central laboratory (Synlab, Heidelberg).

We divided children with CKD into 7 groups according to eGFR (for details, see Figure 6) and calculated the mean (SEM) circulating concentration of each analyte in patients within different eGFR intervals to observe trends in the concentrations of the analytes and the relationship among them with decreasing GFR.

3.2 Laboratory methods

The electrochemiluminescence immunoassay for intact PTH (ECLIA; Roche PTH, Intact [iPTH]) a biotinylated monoclonal antibody reactive with amino acids 26-32 and a capture ruthenium-complexed monoclonal antibody reactive with amino acids 55-64 was used. The measurements were performed on Roche Modular E 170®. (19) The second and third generation PTH assays currently used in clinical practice (also called intact PTH assays) measure both together: oxPTH and n-oxPTH (iPTH = n-oxPTH + oxPTH), which

cannot distinguish between oxidized and non-oxidized PTH. N-oxPTH was measured by a new detection process consists of two steps. In the first step, all oxidized forms of oxPTH at the positions Met8 and/or Met18 are removed from the sample by a specific affinity chromatography column (Immundiagnostik AG, Bensheim, Germany) with a monoclonal antihuman ox-PTH antibody immobilized on Sepharose. These mAbs have shown high specificity against all forms of human oxidised PTH in previous experiments(15). In the second step, the remaining n-oxPTH is measured in a conventional second generation PTH system (Hoffmann-La Roche, Basel, Switzerland). In short, the columns were centrifuged at 3000 rpm for 2 minutes to remove any PBS buffer from the column before 300 ml plasma sample was applied. After sealing, the columns were incubated and mixed end to end for 1 hour at room temperature. The columns were put on a sample tube and centrifuged again at 3000 rpm for 2 minutes to collect the eluate. The eluates were then used for analysis for nox-PTH.

We used the following ELISAs for cFGF23, TRAP5b and sclerostin in the paediatric 4C study(Table 1): cFGF-23 (Immutopics, San Clemente, CA, USA, see: https://www.quidel.com/sites/default/files/product/documents/PI6061001EN00.pdf); TRAP5b (Quidel, CA, USA, see: https://www.tecomedical.com/en/Laboratory-IVD-Kits-Reagents/Bone-and-Cartilage-parameters/Bone-metabolism/TRAP5b-Human-Quidel) ; sclerostin (TECOmedical, Sissach, Switzerland, see: https://www.tecomedical.com/en/Laboratory-IVD-Kits-Reagents/Bone-and-Cartilage-parameters/Bone-metabolism/Sclerostin-TECO-High-sensitive).

Creatinine, albumin, calcium, phosphate, cholesterol, fasting blood glucose, HbA1c, 1,25$(OH)_2$D, and urinary protein were measured using standardized laboratory techniques.

All samples were measured in duplicate and all assays were subject to routine quality control.

3.3 Cell culture

Cell culture and experiments with UMR106 rat osteoblast-like cells (ATCC®, CRL-1661™) were conducted as described by Bär et al. recently(21). Cells were cultured under standard culture conditions in DMEM high-glucose medium containing 10% FBS (Gibco, Life Technologies) and 100 U/mL penicillin/100 µg/ml streptomycin (Gibco, Life Technologies). The cells were first cultured for 24 hours and then treated with or without the various PTH 1-34 derivatives as indicated for another 24 hours. PTH 1-34 derivatives include n-oxPTH, PTH oxidized at Met18 (Met18(ox)-PTH), PTH oxidized at Met8 (Met8(ox)-PTH) as well as PTH oxidized at both methionine amino acids (Met 8 and Met18) (Met8, Met18(di-ox)-PTH), which were purchased from JPT Peptide Technologies GmbH (Volmerstraße 5 (UTZ), 12489 Berlin, Germany).

The quality of the PTH peptides used was proven by HPLC, the HPLC analysis showed the purity of >95% of all peptides. FGF23 and the reference gene mRNA was quantified by qRT-PCR consists of three steps. In the first step, RNA was extracted with peqGOLD TriFast (Peqlab). In the second step, cDNA was synthesized using GoScript Reverse Transcription System (Promega). In the third step, qRT-PCR was performed with GoTaq qPCR Master Mix (Promega) (95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 57 °C (Fgf23, Rpl13a), 60 °C (Sost), 54 °C (Il6), 58 °C (Actb) or 55 °C (G6pd) for 30 s, and then 72 °C for 25 s) using following rat primers: Fgf23: 5´-TAGAGCCTATTCAGACACTTC and 5´-CATCAGGGCACTGTAGATAG; Rpl13a: 5´-GCACAAGACCAAAAGAGG and 5´-CGCTTTTTCTTGTCATAGGG; Sost: 5´-ATGATGCCACAGAAATCATC and 5´-CACGTCTTTGGTGTCATAAG; IL6: 5´-CAGAGTCATTCAGAGCAATAC and 5´-CTTTCAAGATGAGTTGGATGG; Actb: 5´-CGCCACCAGTTCGCCAT and 5´-TACCCACCATCACACCCTGG; G6pd: 5´-ATTGCTTGTGTGATCCTTAC and 5´-CACTTTGACCTTCTCATCAC.

Table 1. Intact PTH, cFGF23, sclerostin and TRAP5b assays.

	Lower limit of detection/upper limit of detection	Additional Assay information's provided by the maufacturer
iPTH (ECLIA; Roche PTH) https://www.roche.de/res/content/9933/_pth_factsheet.pdf	LOD: 5.50 pg/ml; Upper limit of detection: 2300 pg/ml; LLOQ: 10.0 pg/ml	The test only detects biologically intact PTH (1-84).
cFGF-23 (Immutopics, USA) https://www.quidel.com/sites/default/files/product/documents/PI6061001EN00.pdf	LOD: 1.5 RU/mL; Upper limit of detection: the highest concentration of human FGF - 23 measurable without dilution is the value of the highest standard LLOQ: no data	The assay measures both intact FGF23 and C-terminal fragments of FGF23.
sclerostin (TECOmedical, Sissach, Switzerland) https://www.tecomedical.com/en/Laboratory-IVD-Kits-Reagents/Bone-and-Cartilage-parameters/Bone-metabolism/Sclerostin-TECO-High-sensitive	LOD: 0.009 ng/ml Upper limit of detection: 3 ng/ml LLOQ: 0.058 ng/ml	The Human Sclerostin HS Enzyme Immunoassay for the quantitation of Sclerostin in human plasma and serum is a two-step procedure utilizing (1) a microassay plate coated with streptavidin and a biotinylated goat polyclonal antibody that binds specifically to human Sclerostin, (2) a HRP-conjugated monoclonal anti-human Sclerostin antibody, and (3) a chromogenic substrate.
TRAP5b (Quidel, CA, USA) https://www.tecomedical.com/en/Laboratory-IVD-Kits-Reagents/Bone-and-Cartilage-parameters/Bone-metabolism/TRAP5b-Human-Quidel	LOD: 0.2 U/L Upper limit of detection: 15.5 U/L LLOQ: 2.5 U/L	Naturally occurring, inactive TRAP5b fragments in the serum may interfere with the detection of TRAP5b in physiological samples. The Quidel® TRAP5b Assay avoids the influence of the inactive fragments by using two different monoclonal antibodies.

LOD, the lower limit of detection; LLOQ, the lower limit of quantification.

3.4 Statistics

Statistical analyses were performed using SPSS, version 19 (IBM, Armonk, NY, USA) and $p<0.05$ were regarded as statistically significant.

The one-way ANOVA test followed by Dunnett's test was applied for comparison of FGF23**,** G6PD, actin, IL6 and SOST gene expression induced by n-oxPTH- and different forms of ox-PTH-peptides in cultured UMR106 cells. The statistical difference in the mean value of the individual parameters of the three patient groups (CKD stage 1-2, stage 3 and stage 4-5) of the paediatric 4C population was analyzed using one-way ANOVA. The values of the variables with non-normal distribution were ln transformed. The correlation of iPTH and cFGF23 with oxidized or non-oxidized PTH respectively were performed by the linear correlation analysis. The correlation between FGF23 and either oxPTH or n-oxPTH in the clinical studies were investigated by linear multivariate regression models considering known confounding factors for FGF23 syntheses. We used Spearman's rho to assess bivariate associations of CKD-MBD biomarkers, sclerostin and TRAP5b with iPTH, n-oxPTH and oxPTH in children with CKD.

4. Results

4.1 In vitro studies

4.1.1 Effect of n-oxPTH and oxidized PTH-derivatives on FGF23 expression in cultured cells.

UMR106 osteoblast-like cells were cultured with n-oxPTH and various forms of oxidized PTH to determine the effect of PTH oxidation on the transcriptional regulation of FGF23. We performed a dose-response curve to investigate the following concentrations of n-oxPTH and different forms of oxidized PTH in the cell culture medium: 3, 10, 30, 100 and 300 nmol/l. (Figure 2) All investigated dosages of n-oxPTH stimulated the expression of FGF23 mRNA. Met18 oxPTH has a partial agonistic effect on the synthesis of FGF23 mRNA. Neither Met8 oxPTH nor Met8, Met18 di-oxPTH led to a significant stimulation of FGF23 mRNA synthesis at any of the investigated dosages.

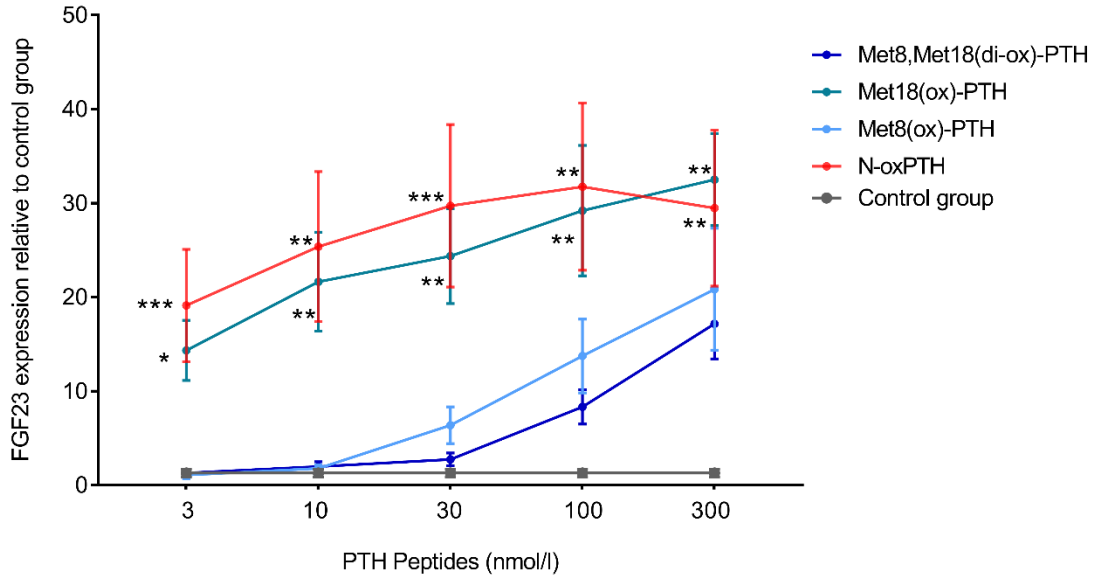


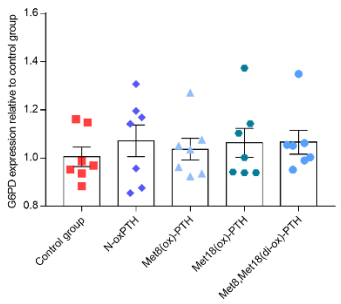
Figure 2. Effect of non-oxidized PTH (n-oxPTH) and different forms of oxidized PTH on FGF23 expression in cultured UMR106 osteoblast-like cells.
The cells were first cultured for 24 hours and then treated with or without the addition of different dosages (3, 10, 30, 100 and 300 nmol/l) of the various PTH 1-34 derivatives as indicated for another 24 hours. FGF23 mRNA expression (relative to RPL13a) was measured by qRT-PCR. The experiment was repeated 7 times. All investigated dosages of n-oxPTH and Met18 oxPTH stimulated the expression of FGF23 mRNA. Neither Met8 oxPTH nor Met8, Met18 di-oxPTH led to a significant stimulation of FGF23 mRNA synthesis at any of the treated dosages. (Dunnett's test) The x axis represents the concentration of PTH-peptides. The y axis represents FGF23 expression relative to control group. This figure is adapted from our published paper.(19)

4.1.2 Effect of n-oxPTH and oxidized PTH-derivatives on G6PD, actin, IL6 and SOST expression in cultured cells.

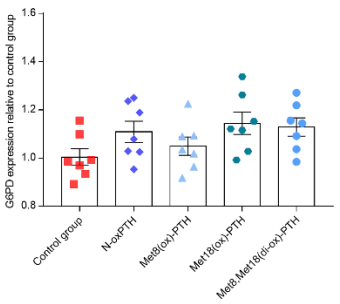
Beside the experiments on the stimulation of FGF23 gene expression by different PTH-peptides, we also did the experiments with negative (actin and glucose-6-phosphate-dehydrogenase) and positive (IL-6 and sclerostin,(22-25)) controls in UMR106 cells, see Figure 3. The expression of the negative controls (actin and glucose-6-phosphate-dehydrogenase) was not influenced by any dose of the PTH peptides (3 and 10 nmol/L). However, it is of interest the positive control behaved like FGF23, non-oxidized PTH does stimulate IL-6. Met18(ox)-PTH was also stimulating the expression of IL-6, whereas Met8(ox)-PTH and Met8, Met18(di-ox)-PTH did not stimulate IL-6. Exactly the same

pattern was seen for the stimulation of FGF23. N-oxPTH and Met18(ox)-PTH also suppresses the expression of sclerostin, whereas Met8(ox)-PTH and Met8, Met18(di-ox)-PTH did not alter the expression of sclerostin.

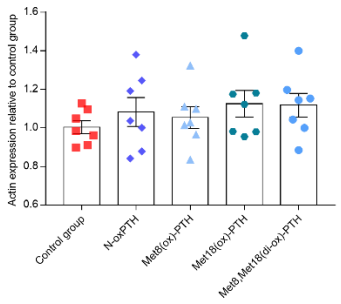
(a)G6PD (PTH Peptides: 3nmol/l)



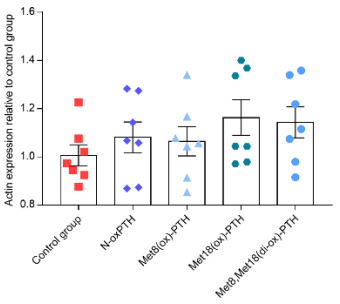
(b)G6PD (PTH Peptides: 10nmol/l)



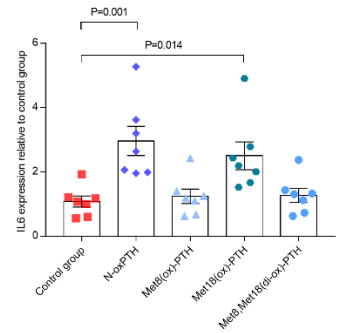
(c)Actin (PTH Peptides: 3nmol/l)



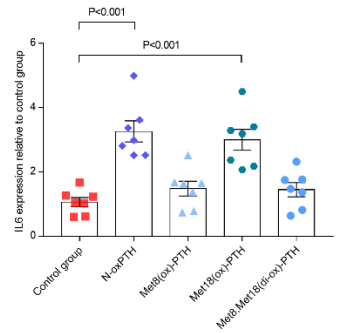
(d)Actin (PTH Peptides: 10nmol/)l



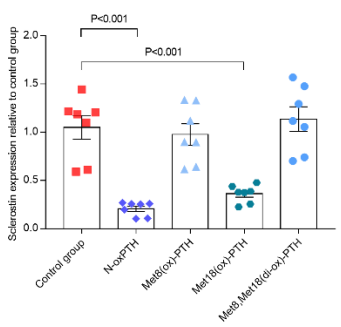
(e)IL6 (PTH Peptides: 3nmol/l)



(f)IL6 (PTH Peptides: 10nmol/l)



(g)SOST (PTH Peptides: 3nmol/l)



(h)SOST (PTH Peptides: 10nmol/l)

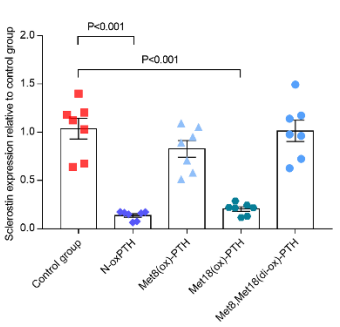


Figure 3. Effect of non-oxidized PTH (n-oxPTH) and different forms of oxidized PTH on G6PD, actin, IL6 and SOST expression in cultured UMR106 osteoblast-like cells. Cells were first grown for 24 h and then treated with or without the addition of two dosages (3 and 10nmol/l) of the different PTH 1-34 derivatives as indicated for additional 24 h. Glucose-6-phosphate-dehydrogenase (G6PD), actin, Interleukin 6 (IL6) and SOST gene expression (relative to RPL13a) was measured by qRT-PCR. The experiment was repeated 7 times.
(a, b, c & d). Neither n-oxPTH nor different forms of oxidized PTH led to a significant stimulation of G6PD and actin mRNA synthesis at any of the investigated dosages (Dunnett's test).
(e, f, g & h) Two dosages of n-oxPTH and Met18 oxPTH led to a significant stimulation of IL6 and SOST mRNA synthesis. Neither Met8, Met18 di-oxPTH nor Met8 oxPTH caused a significant stimulation of IL6 and SOST mRNA expression at any of the investigated dosages. The x axis denotes the concentration. The y axis denotes relative FGF23 expression. This figure is adapted from our published paper.(19)

4.2 Patients cohorts

4.2.1 Testing of the time stability of the n-oxPTH assay

We have recently shown that the affinity column can completely remove oxidized PTH(15), controlled by liquid chromatography-mass spectrometry (LC-MS). Figure 4 shows that n-oxPTH values measured after our anti-oxPTH column are stable over a wide range of times (10 min to 120 min) on column (60 min in the standard time for the assay used in this study). This also rules out “column-induced oxidation” which means an oxidation of PTH on the column. It is of note that the rate of oxidation is lower in the healthy controls. The intrinsic difference in oxidation levels of PTH between healthy and CKD plasma specimens remains stable.

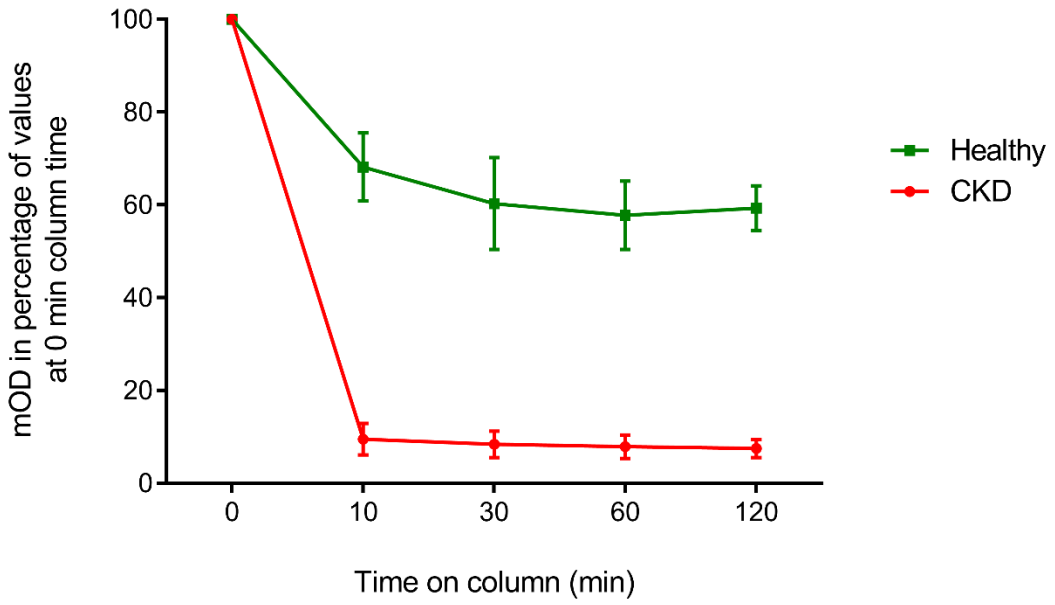


Figure 4. N-oxPTH independent of time on column and intrinsically different in healthy and CKD samples.
Plasma of CKD patients (n=8, mean iPTH: 394.1±200.7) and healthy volunteers (n=8, mean iPTH: 22.9±8.4) was incubated on the anti-oxPTH column for various time intervals, followed by a conventional iPTH sandwich assay. Data (mean ± SD) is given in mOD in percentage of the values at 0 min column time. N-oxPTH values measured after our anti-oxPTH column are stable over a wide range of times (10 min to 120 min) on column (60 min in the standard time for the assay used in this study). Modifications adopted from our published paper(19).

4.2.2 Patient characteristics

A total of 620 children with CKD, 216 females (34.8%) and 404 males (65.2%) were enrolled in this study. The baseline characteristics are presented in Table 2. Twenty-one patients showed a higher than required GFR (> 60ml/min/1.73m^2) after recruitment than during the screening visit, but were retained in the study (CKD stage 1-2).

At study entry, mean (SD) age of patients was 12.1(3.3) years, and the mean height standard deviation score was -1.4(1.5). The mean estimated glomerular filtration rate (eGFR) was 32.8(13.3) mL/ min/1.73m^2. The average plasma concentrations of oxPTH, n-oxPTH and iPTH were146.5 (160.8) pg/ml (n=618), 17.4(14.6) pg/ml (n=618) and 163.5(172.3) pg/ml (n=620), respectively. We used cFGF23 data from the 4C study(26) which are measured only in patients with a GFR < 60 mL/min/1.73 m^2; the mean

concentration of cFGF23 was 322.4(491.6) pg/ml(n=552).

Table 2. Patient characteristics of children with CKD (n=620).

	All	Stages 1-2	Stages 3	Stages 4-5	P
N	620	21	296	298	
Sex (female/male)	216 f/404 m	7 f/14 m	93 f/203 m	114 f/184 m	
Age at study entry (years)	12.1(3.3)	11.7(2.9)	12.2(3.3)	12.1(3.4)	0.691
Height standard deviation score	-1.4(1.5)	-1.1(1.7)	-1.1(1.5)	-1.6(1.4)	<0.001
Cystatin C (mg/L)	2.8(0.9)	1.4(0.3)	2.3(0.4)	3.5(0.8)	<0.001
eGFR (mL/min/1.73 m^2)	32.8(13.3)	72.6(14.3)	40.3(7.5)	22.5(4.6)	<0.001
Plasma calcium(mmol/L)	2.2(0.2)	2.2(0.3)	2.2(0.2)	2.2(0.2)	0.839
Plasma phosphate (mmol/L)	1.5(0.4)	1.5(0.6)	1.4(0.4)	1.6(0.4)	<0.001
Sclerostin (ng/ml)	0.32(0.14)	0.31(0.14)	0.31(0.16)	0.33(0.15)	0.225
TRAP5b (U/L)	12.7(6.5)	13.0(6.2)	11.7(5.9)	12.6(6.9)	0.587
BAP (mcg/L)	150.0(84.8)	134.3(78.0)	146.5(77.1)	155.3(92.5)	0.315
Plasma creatinine (mg/dl)	2.6(1.4)	0.9(0.2)	1.8(0.5)	3.6(1.4)	<0.001
OxPTH (pg/ml)	146.5(160.8)	46.1(51.3)	110.4(118.0)	190.0(188.9)	<0.001
N-oxPTH (pg/ml)	17.4(14.6)	13.8(23.2)	13.6(11.2)	21.5(15.8)	<0.001
Intact PTH (pg/ml)	163.5(172.3)	59.9(44.9)	123.5(126.7)	211.5(202.4)	<0.001
C-terminal FGF23 (pg/ml)	322.4(491.6)		204.6(321.5)	444.4(598.2)	<0.001

Data are given as mean (SD) or n. The 4C study provides cFGF23 data without giving reasons only for patients with GFR <60 mL/min/1.73 m^2. There are 5 patients without GFR data. The statistical difference in the mean value of the individual parameters of the three patient groups (CKD stage 1-2, stage 3 and stage 4-5) of the the paediatric 4C population was analyzed using one-way ANOVA. The statistical difference in mean C-terminal FGF23 between patients with stage 3 and stage 4-5 CKD was analyzed by two independent T-test. Patients were categorized according to CKD stages as described.(27, 28) BAP, bone alkaline phosphatase; TRAP5b, tartrate-resistant acid phosphatase 5b. Modifications adopted from our published paper(19).

4.2.3 The correlation between cFGF23 and oxidized or non-oxidized PTH

To translate the cell experiment findings showing that n-oxPTH stimulates the expression of FGF23 gene in vitro into clinical science, we investigated the relationship of FGF23 and either n-oxPTH or oxPTH in 620 children with CKD. Linear correlation analysis indicated that C-terminal FGF23 plasma concentrations are significantly positively correlated with n-oxPTH (Figure 5, $p<0.001$), but not with oxPTH ($p=0.0514$).

Since the secretion of FGF23 is also influenced by many other factors, we have considered the following confounding factors in a multivariate linear regression analysis: patient age, score of the height standard deviation, GFR stage, calcium, and phosphate. The results showed that the concentration of cFGF23 was independently associated with n-oxPTH (95% CI 0.011 - 0.236; $p=0.0313$) but not with oxPTH (95% CI -0.074 - 0.076; $p=0.9742$). (Table 3) Intact PTH – the sum of n-oxPTH and oxPTH –was also not correlated with C-terminal FGF23 (Table 4).

Taken together, the linear correlation analysis between hormone concentrations (Figure 5) as well as the multivariate linear regression analysis (Table 3) show that n-oxPTH, but not oxPTH, is positively and independently of known confounders associated with FGF23 in children with CKD.

Table 3. Multivariate linear regression of variables associated with C-terminal FGF23 plasma concentrations in children with CKD.

	Standardized Beta	T	P	95% Confidence Interval for B
Contains oxPTH				
Age	-0.015	-0.380	0.704	-0.023 - 0.016
Height standard deviation score	0.189	4.862	<0.001	0.066 - 0.156
GFR stage	0.409	10.260	<0.001	0.512 - 0.754
Calcium	0.052	1.285	0.199	-0.093 - 0.445
Phosphate	0.184	4.588	<0.001	0.242 - 0.604
OxPTH	0.001	0.032	0.974	-0.074 - 0.076
Contains n-oxPTH				
Age	-0.017	-0.437	0.662	-0.024 - 0.015
Height standard deviation score	0.195	5.036	<0.001	0.069 - 0.158
GFR stage	0.384	9.426	<0.001	0.470 - 0.718
Calcium	0.070	1.799	0.073	-0.022 - 0.498
Phosphate	0.169	4.277	<0.001	0.211 - 0.569
N-oxPTH	0.090	2.159	0.031	0.011 - 0.236

GFR stage: stage 1: eGFR ≥ 90 mL/min/1.73m^2; stage 2: eGFR 60-89 mL/min/1.73 m^2; stage 3: eGFR 30-59 mL/min/1.73m^2; stage 4: eGFR 15-29 mL/min/1.73 m^2; stage 5: eGFR <15 mL/min/1.73m^2. OxPTH, oxidized parathyroid hormone; n-oxPTH, non-oxidized parathyroid hormone. Modifications adopted from our published paper(19).

Table 4. Multivariate linear regression of variables associated with C-terminal FGF23 plasma concentrations in children with CKD.

	Standardized Beta	T	P	95% Confidence Interval for B
Age	-0.018	-0.470	0.638	-0.024-0.015
Height standard deviation score	0.189	4.882	<0.001	0.066-0.155
GFR stage	0.407	10.197	<0.001	0.508-0.750
Calcium	0.055	1.377	0.169	-0.080-0.456
Phosphate	0.180	4.508	<0.001	0.234-0.595
Intact PTH	0.020	0.463	0.644	-0.061-0.099

GFR stage: stage 1: eGFR ≥ 90 mL/min/1.73m^2; stage 2: eGFR 60-89 mL/min/1.73 m^2; stage 3: eGFR 30-59 mL/min/1.73m^2; stage 4: eGFR 15-29 mL/min/1.73 m^2; stage 5: eGFR <15 mL/min/1.73m^2. Modifications adopted from our published paper(19).

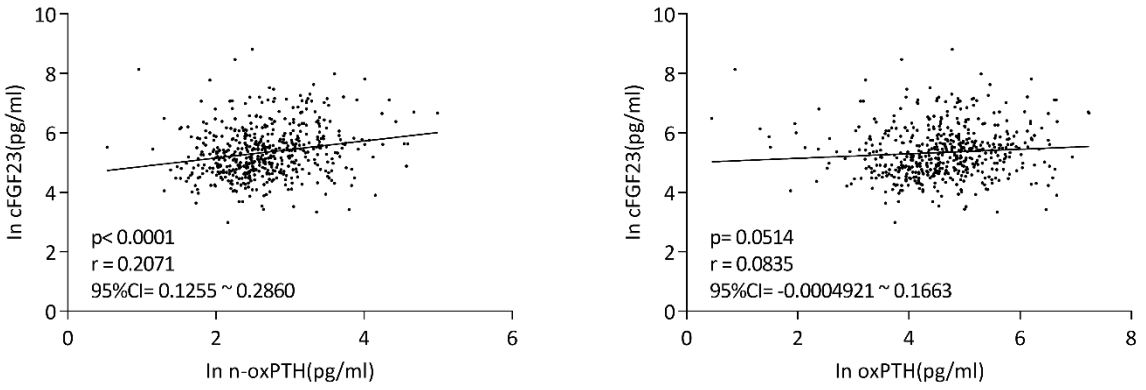


Figure 5. The correlation between cFGF23 and oxidized or non-oxidized PTH.
C-terminal FGF23 plasma concentrations are positively correlated with n-oxPTH, but not oxPTH in children with CKD. Modifications adopted from our published paper(19).

4.2.4 Correlation of iPTH, n-oxPTH, oxPTH, and sclerostin, TRAP5b

In addition to analyzing the correlation of FGF23 and various PTH peptides, we also performed an evaluation of other circulating CKD-MBD biomarkers, sclerostin and TRAP5b, and their relationship to iPTH, n-oxPTH and oxPTH. The results showed that both were correlated with n-oxPTH and oxPTH. (Table 5)

Table 5. Correlation of iPTH, n-oxPTH, oxPTH, and sclerostin, TRAP5b in children with CKD at study entry.

		intact PTH	n-oxPTH	oxPTH
Sclerostin	r_s	-0.102	-0.142	-0.141
	p	0.014	0.001	0.001
	n	588	585	590
TRAP5b	r_s	0.192	0.228	0.229
	p	<0.001	<0.001	<0.001
	n	598	595	600

We used Spearman's rho to evaluate bivariate associations. TRAP5b, tartrate-resistant acid phosphatase 5b. Modifications adopted from our published paper(19).

4.2.5 The correlation between intact PTH and oxidized or non-oxidized PTH

The linear correlation analysis demonstrated a close to linear correlation between iPTH and oxPTH (r^2 =0.9938; p < 0.0001), but a much weaker correlation between iPTH and n-oxPTH (r^2 = 0.74; p < 0.0001; Figure 6).

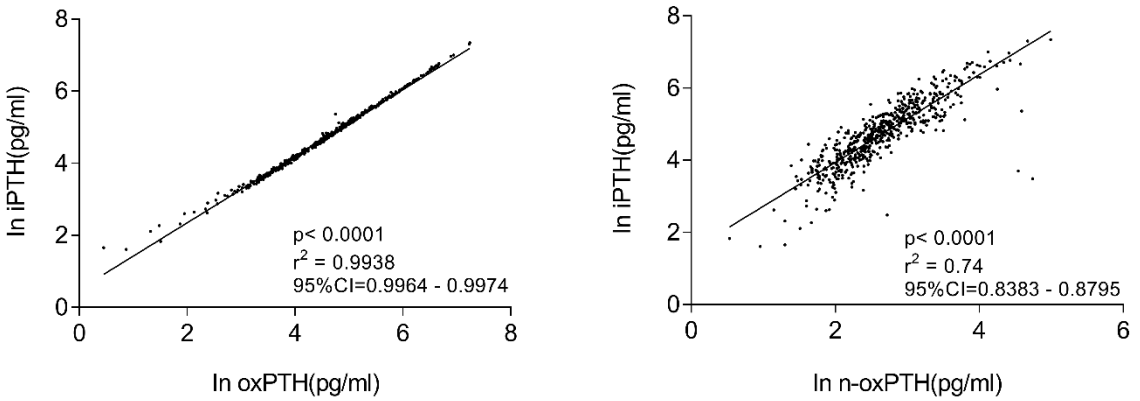


Figure 6. The correlation between intact PTH and oxidized or non-oxidized PTH. Intact PTH correlates very well with oxPTH but to a much weaker extent with n-oxPTH.

4.2.6 Plasma levels of cFGF23 and different forms of PTH according to eGFR

In the 4C study population, with the decrease in eGFR, iPTH and oxPTH started to increase substantially in early stage CKD, while n-oxPTH increased only moderately in late stage CKD. (Figure 7) From CKD stage 1-2 to stage 4-5, iPTH increased from 59.9 (44.9) pg/mL to 211.5 (202.4) pg/mL. The elevation of iPTH (151.6 pg/mL) is mainly due to an increase in oxPTH (+143.9 pg/mL), while n-oxPTH (+7.7 pg/mL) contributed only slightly to this iPTH increase. (table 2)

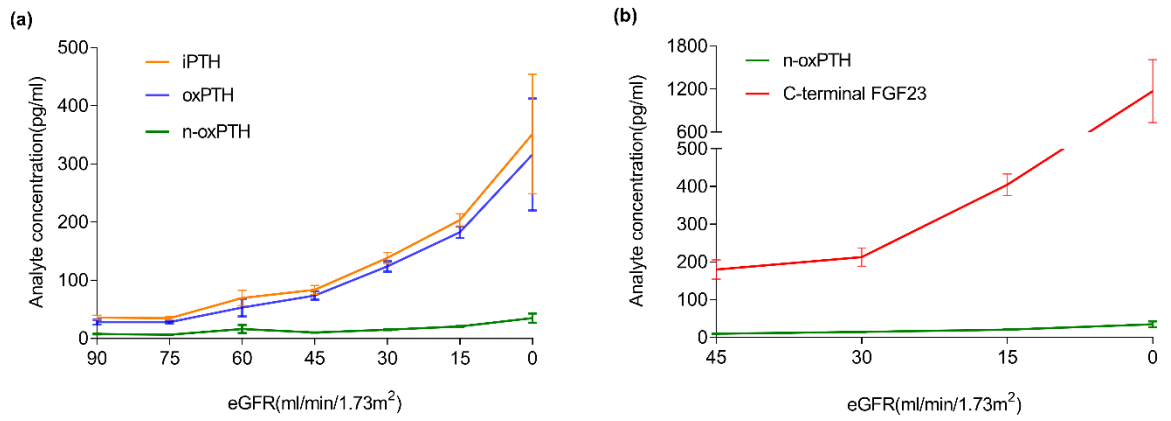


Figure 7. Plasma levels of C-terminal FGF23, iPTH, non-oxidized and oxidized forms of PTH according to increments of eGFR in children with CKD.
The patients were divided into 7 groups based on the eGFR and calculated the mean (SEM) concentrations of each analyte in circulation in patients with various intervals of the eGFR, respectively. 90 represents eGFR > 90 ml/min/1.73m^2; 75 represents eGFR > 75 and ≤ 90 ml/min/1.73m^2; 60 represents eGFR > 60 and ≤ 75 ml/min/1.73m^2 ; 45 represents eGFR > 45 and ≤ 60 ml/min/1.73m^2; 30 represents eGFR > 30 and ≤ 45 ml/min/1.73m^2; 15 represents eGFR > 15 and ≤ 30 ml/min/1.73m^2; 0 represents eGFR > 0 and ≤ 15ml/min/1.73m^2. The y axis represents the average circulating concentrations of the individual analytes (n-oxPTH [pg/ml] in green; oxPTH [pg/ml] in blue; iPTH [pg/ml] in orange and FGF23 [pg/ml] in red). The concentration of n-oxPTH is 4.6 times higher, when eGFR is 0-15 ml/min/1.73m^2 compared to an eGFR of over 90 ml/min/1.73m^2 (P=0.0043). We used cFGF23 data from the 4C study(26) measured only in patients with a GFR < 60 mL/min/1.73 m^2. Modifications adopted from our published paper(19).

5. Discussion

5.1 Summary of the important findings

This study confirms different biological functions of non-oxidized and oxidized forms of PTH (Figure 8). While n-oxPTH stimulated the synthesis of FGF23 mRNA in vitro, the oxidation of PTH led to a significantly weaker stimulation of FGF23 mRNA synthesis, especially at the position Met8. In the 4C study population, FGF23 concentrations was only associated with n-oxPTH, but not with oxPTH, regardless of known confounding factors. With progressive deterioration of renal function, concentrations of FGF23 and iPTH increased substantially, while the biologically active n-oxPTH increased far less than iPTH and oxPTH.

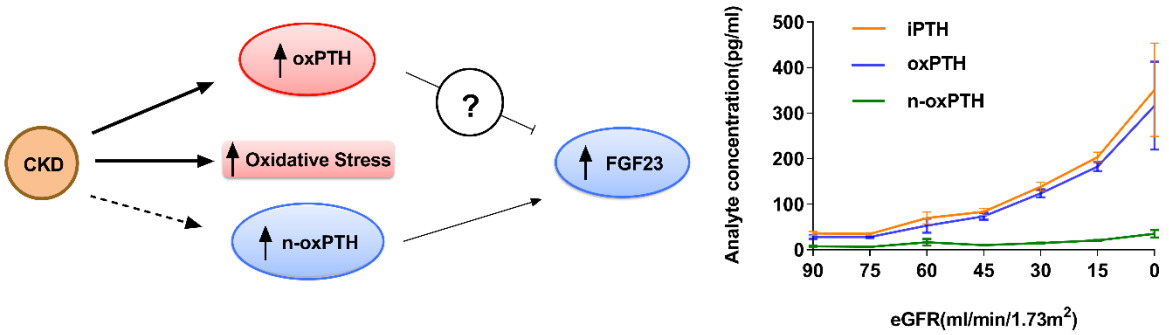


Figure 8. A graphical abstract of the main findings of the present study
N-oxPTH, but not oxPTH stimulates the synthesis of FGF23 in vitro. The results of the 4C cohort study are consistent with this finding and show an independent association of FGF23 with n-oxPTH, but not with oxPTH. With decreasing GFR, n-oxPTH increases only moderately. The increase in PTH reported in the literature - measured by iPTH assays - with decreasing GFR is mainly due to an elevation of oxPTH. Modifications adopted from our published paper(19).

5.2 N-oxPTH but not oxPTH stimulates FGF23

The cell culture experiments showed that n-oxPTH 1-34 stimulates the synthesis of FGF23 mRNA much stronger than oxPTH 1-34, which is oxidized especially at the position Met8. These findings are in agreement with the literature. The parathyroid hormone/thyroid hormone-related protein receptor (PTH/PTHrP type 1 receptor, also

known as PTHR1) is a member of the family of G-protein coupled receptors (GPCR), which is primarily expressed in kidney, cartilage and bone, but is also expressed in other tissues including blood vessels. (29) Studies on the structure-activity relationship of ligands and receptor mutagenesis suggest that bioactive PTH (1-34) interacts with PTHR1 through a two-component mechanism. The C-terminal portion of PTH(1–34) interacts with the amino-terminal extracellular domain of PTHR1 (site 1), while the N-terminal portion interacts with extracellular connecting loops and the transmembrane helices (site 2).(29) The interactions with site 1 provide the majority of the energetic drive for binding, whereas contacts with site 2 induce the conformational changes in the receptor that initiate intracellular signaling.

Two methionine amino acids at positions 8 and 18 of PTH molecule can be oxidized in vivo and there is overwhelming evidence that n-oxPTH and oxPTH have different biological properties. These results are reflected in the following three lines of evidence:

a) Initial studies focused on the binding affinity to the PTH receptor and the generation of the second messenger of the PTH receptor, cAMP. It has been shown that oxPTH has a much lower binding affinity to the PTH receptor in classical receptor binding assays. (13) In contrast to n-OxPTH, OxPTH does not stimulate the PTH receptor to generate cAMP. (13)
b) OxPTH loses its biological effect on smooth muscle cell/vascular contraction in tissues such as the uterus, renal tubules, trachea, and aortic rings. Only after incubation with n-oxPTH, but not with oxPTH, can the stimulation of alkaline phosphatase activity by PTH be observed in cultured neonatal cranial bone cells of mice. Other studies demonstrate that only n-oxPTH -but not oxPTH- is able to regulate the metabolism of calcium and phosphate in vivo. For further details see the review of these studies in Hocher et al. (13)
c) Our finding that the effect of oxidation at methionine 8 seems to be more critical for the effects of PTH on its receptor is well consistent with structural analysis of oxidized PTH peptides. The study by Zull et al(30) showed that the oxidation of the methionine residue at position 8 causes significant changes in the secondary

structure, whereas the oxidation of the methionine at position 18 has only a minor effect. Oxidation of both positions produces secondary structure changes that are greater than the sum of those seen upon oxidation of the individual positions.(30) Another study showed that oxidation at methionine 8 was more potent than at methionine 18 to inhibit the formation of the second messenger of PTH, cAMP(31), which is in line with our data and the data by Zull et al.(30).

5.3 The correlation between cFGF23 and oxidized or non-oxidized PTH

A positive correlation of n-oxPTH - but not oxPTH - with FGF23 independent of known confounding factors was observed in the 4C study cohort.

Previous in vitro and in vivo studies investigating the relationship between PTH and FGF23 had contradictory results. For example, an increase in the expression of FGF23 mRNA from murine osteocytes in vitro by activation of the PTHR1 receptor has been demonstrated(6, 32), while some animal experiments showed a decrease in plasma FGF23 concentration during PTH treatment(33-35).

In humans, Burnett-Bowie et al. (36) demonstrated that a PTH infusion in healthy men increased FGF23 levels over 18 h(36), whereas Guiterrez and colleagues(37) showed an acute decrease in FGF23 levels with a PTH infusion within 6 h. The reason for these inconsistent results is not fully understood and may partly be due to differences in the populations studied. We have previously described the existence of high concentrations of ox-PTH in the circulation of CKD patients(13); here we show significant differences in comparison to n-oxPTH concentrations in their associations with FGF23 and GFR, which further confirms their different biological properties in vivo.

In contrast to the present literature on the relationship between PTH and decreasing GFR(3, 38), biologically active n-oxPTH increased significantly less than iPTH and oxPTH as renal function deteriorated. These results suggest that oxidation of PTH increases progressively with the decrease of GFR, reflecting the extent of oxidative stress as described by others.(39)

These data indicate that the increase in PTH with the decrease of GFR described in

textbooks on renal physiology primarily reflects the increase in oxPTH, but not n-oxPTH. Our data suggest that the progressive increase of FGF23 with decreasing GFR does not appear to be related to the moderate increase of n-oxPTH, but that other factors may play a more important role in regulating the concentration of FGF23 in CKD. (40)

5.4 The correlation between intact PTH and oxidized or non-oxidized PTH

The linear correlation analysis demonstrated a close to linear correlation between iPTH and oxPTH, but a much weaker correlation between iPTH and n-oxPTH. It suggests that the currently used iPTH assays primarily describe oxidative stress in CKD patients but not PTH bioactivity – for which these iPTH assays were originally developed.

5.5 Study limitations

This study had several limitations that should be considered when interpreting the results. Firstly, our study pointed out that the effect of n-oxPTH on FGF23 is CKD-stage dependent. However, it needs be confirmed by injection of n-oxPTH at different stages of CKD in experimental animal models. Secondly, as the assay used for oxPTH in the clinical study cannot discriminate between the different forms of oxPTH, we were unable to analyze the relationship between FGF23 and each form of oxPTH and their respective trends with decreasing GFR. There is a need for a clinically suitable measurement system that can distinguish between different forms of PTH. Given the enormous clinical impact of PTH, our research may stimulate the development of such methods. Finally, the extent of oxidative stress and its effects on circulating concentrations of FGF23 and PTH could not be assessed because no markers of oxidative stress were measured in the clinical studies. In this context, it is quite important to know that PTH oxidation occurs in vivo and is largely independent of sample handling(14) and appears to be influenced by vitamin D(41).

5.6 Expand

We suspect that our research is of universal importance, far beyond understanding the PTH biological properties. It could suggest that the oxidation of peptide hormones alters the biological properties of these hormones. Many hormones contain methionine in their amino acid sequence. This could have similar consequences as shown for PTH.

The use of iPTH measurements to identify patients with secondary hyperparathyroidism, as previously conducted in the EVOLVE trial, could lead to the selection of patients without true secondary hyperparathyroidism(42) and therefore contribute to the failure of such clinical trials, although the drug under investigation is potentially effective. The data provided here, as well as published clinical data on the topic(13, 14, 43-47), make the emergence of iPTH analysis for clinical purposes critical. It may currently be more appropriate to measure bone alkaline phosphatase for clinical use until further research allows the guidelines for n-oxPTH to be established. Oxidized PTH is more likely to be a biomarker of oxidative stress. Oxidative stress is for sure also harmful to CKD patients and will further promote the progression of the disease. However, the treatment of oxidative stress is certainly different from the treatment of PTH disorders in CKD patients. The majority of PTH is present in oxidized form and has no biological activity. Only assay systems that measure bioactive PTH (n-oxPTH) are capable of correctly guiding clinical decision making. However, PTH assays designed for clinical use should measure bioactive PTH since clinicians need to correctly adjust medications such as calcimimetics, phosphate binders or vitamin D based on this information. All of these drugs are essential for patients with CKD and can affect the endocrine PTH system. The use of a clinical laboratory instrument that cannot actually measure bioactive PTH makes clinical decision making for patients quite difficult or even risky.

5.7 Conclusion

Only n-oxPTH, but not oxPTH, stimulates the synthesis of FGF23 in vitro. The results of our cross-sectional cohort study are consistent with this finding showing an independent association between FGF23 and n-oxPTH, but not oxPTH. Furthermore, we could

demonstrate that with decreasing GFR, n-oxPTH increases only moderately. The increase in PTH reported in the literature - measured by iPTH assays - with decreasing GFR is mainly due to an elevation of oxPTH.

6. References

1. Erben, R. G. (2018) Physiological Actions of Fibroblast Growth Factor-23. *Front Endocrinol (Lausanne)* **9**, 267
2. Vervloet, M. (2019) Renal and extrarenal effects of fibroblast growth factor 23. *Nat Rev Nephrol* **15**, 109-120
3. Isakova, T., Wahl, P., Vargas, G. S., Gutierrez, O. M., Scialla, J., Xie, H. L., Appleby, D., Nessel, L., Bellovich, K., Chen, J., Hamm, L., Gadegbeku, C., Horwitz, E., Townsend, R. R., Anderson, C. A. M., Lash, J. P., Hsu, C. Y., Leonard, M. B., Wolf, M., and Grp, C. S. (2011) Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int* **79**, 1370-1378
4. Graciolli, F. G., Neves, K. R., Barreto, F., Barreto, D. V., dos Reis, L. M., Canziani, M. E., Sabbagh, Y., Carvalho, A. B., Jorgetti, V., Elias, R. M., Schiavi, S., and Moyses, R. M. A. (2017) The complexity of chronic kidney disease-mineral and bone disorder across stages of chronic kidney disease. *Kidney Int* **91**, 1436-1446
5. Isakova, T., Cai, X., Lee, J., Xie, D., Wang, X., Mehta, R., Allen, N. B., Scialla, J. J., Pencina, M. J., Anderson, A. H., Talierco, J., Chen, J., Fischer, M. J., Steigerwalt, S. P., Leonard, M. B., Hsu, C. Y., de Boer, I. H., Kusek, J. W., Feldman, H. I., Wolf, M., and Chronic Renal Insufficiency Cohort Study, I. (2018) Longitudinal FGF23 Trajectories and Mortality in Patients with CKD. *J Am Soc Nephrol* **29**, 579-590
6. Lavi-Moshayoff, V., Wasserman, G., Meir, T., Silver, J., and Naveh-Many, T. (2010) PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. *Am J Physiol Renal Physiol* **299**, F882-889
7. Masuyama, R., Stockmans, I., Torrekens, S., Van Looveren, R., Maes, C., Carmeliet, P., Bouillon, R., and Carmeliet, G. (2006) Vitamin D receptor in chondrocytes promotes osteoclastogenesis and regulates FGF23 production in osteoblasts. *J Clin Invest* **116**, 3150-3159
8. David, V., Martin, A., Isakova, T., Spaulding, C., Qi, L. X., Ramirez, V., Zumbrennen-Bullough, K. B., Sun, C. C., Lin, H. Y., Babitt, J. L., and Wolf, M. (2016) Inflammation and functional iron deficiency regulate fibroblast growth factor 23 production. *Kidney Int* **89**, 135-146
9. Ferrari, S. L., Bonjour, J. P., and Rizzoli, R. (2005) Fibroblast growth factor-23 relationship to dietary phosphate and renal phosphate handling in healthy young men. *J Clin Endocr Metab* **90**, 1519-1524
10. Rodriguez-Ortiz, M. E., Lopez, I., Munoz-Castaneda, J. R., Martinez-Moreno, J. M., Ramirez, A. P., Pineda, C., Canalejo, A., Jaeger, P., Aguilera-Tejero, E., Rodriguez, M., Felsenfeld, A., and Almaden, Y. (2012) Calcium Deficiency Reduces Circulating Levels of FGF23. *J Am Soc Nephrol* **23**, 1190-1197
11. Feger, M., Hase, P., Zhang, B., Hirche, F., Glosse, P., Lang, F., and Foller, M. (2017) The production of fibroblast growth factor 23 is controlled by TGF-beta2. *Sci Rep* **7**, 4982
12. Meir, T., Durlacher, K., Pan, Z., Amir, G., Richards, W. G., Silver, J., and Naveh-Many, T. (2014) Parathyroid hormone activates the orphan nuclear receptor Nurr1 to induce FGF23 transcription. *Kidney Int* **86**, 1106-1115
13. Hocher, B., Oberthur, D., Slowinski, T., Querfeld, U., Schaefer, F., Doyon, A., Tepel, M., Roth, H. J., Gron, H. J., Reichetzeder, C., Betzel, C., and Armbruster, F. P. (2013) 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 & blood pressure research* **37**, 240-251

14. Ursem, S. R., Vervloet, M. G., Hillebrand, J. J. G., de Jongh, R. T., and Heijboer, A. C. (2018) Oxidation of PTH: in vivo feature or effect of preanalytical conditions? *Clin Chem Lab Med* **56**, 249-255
15. Hocher, B., Armbruster, F. P., Stoeva, S., Reichetzeder, C., Gron, H. J., Lieker, I., Khadzhynov, D., Slowinski, T., and Roth, H. J. (2012) Measuring parathyroid hormone (PTH) in patients with oxidative stress--do we need a fourth generation parathyroid hormone assay? *PLoS One* **7**, e40242
16. Hocher, B., and Zeng, S. (2018) Clear the Fog around Parathyroid Hormone Assays: What Do iPTH Assays Really Measure? *Clin J Am Soc Nephrol* **13**, 524-526
17. Panah, F., Ghorbanihaghjo, A., Argani, H., Zarmehri, M. A., and Ahmad, S. N. S. (2018) Ischemic acute kidney injury and klotho in renal transplantation. *Clin Biochem* **55**, 3-8
18. Querfeld, U., Anarat, A., Bayazit, A. K., Bakkaloglu, A. S., Bilginer, Y., Caliskan, S., Civilibal, M., Doyon, A., Duzova, A., Kracht, D., Litwin, M., Melk, A., Mir, S., Sozeri, B., Shroff, R., Zeller, R., Wuhl, E., and Schaefer, F. (2010) The Cardiovascular Comorbidity in Children with Chronic Kidney Disease (4C) study: objectives, design, and methodology. *Clin J Am Soc Nephrol* **5**, 1642-1648
19. Zeng, S., Querfeld, U., Feger, M., Haffner, D., Hasan, A. A., Chu, C., Slowinski, T., Bernd Dschietzig, T., Schafer, F., Xiong, Y., Zhang, B., Rausch, S., Horvathova, K., Lang, F., Karl Kramer, B., Foller, M., and Hocher, B. (2020) Relationship between GFR, intact PTH, oxidized PTH, non-oxidized PTH as well as FGF23 in patients with CKD. *FASEB J*
20. Schwartz, G. J., Munoz, A., Schneider, M. F., Mak, R. H., Kaskel, F., Warady, B. A., and Furth, S. L. (2009) New equations to estimate GFR in children with CKD. *J Am Soc Nephrol* **20**, 629-637
21. Bar, L., Hase, P., and Foller, M. (2019) PKC regulates the production of fibroblast growth factor 23 (FGF23). *PLoS One* **14**, e0211309
22. Lowik, C. W., van der Pluijm, G., Bloys, H., Hoekman, K., Bijvoet, O. L., Aarden, L. A., and Papapoulos, S. E. (1989) Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. *Biochem Biophys Res Commun* **162**, 1546-1552
23. Sakagami, Y., Girasole, G., Yu, X. P., Boswell, H. S., and Manolagas, S. C. (1993) Stimulation of interleukin-6 production by either calcitonin gene-related peptide or parathyroid hormone in two phenotypically distinct bone marrow-derived murine stromal cell lines. *J Bone Miner Res* **8**, 811-816
24. Kramer, I., Loots, G. G., Studer, A., Keller, H., and Kneissel, M. (2010) Parathyroid hormone (PTH)-induced bone gain is blunted in SOST overexpressing and deficient mice. *J Bone Miner Res* **25**, 178-189
25. Yang, H., Dong, J., Xiong, W., Fang, Z., Guan, H., and Li, F. (2016) N-cadherin restrains PTH repressive effects on sclerostin/SOST by regulating LRP6-PTH1R interaction. *Ann N Y Acad Sci* **1385**, 41-52
26. Doyon, A., Fischer, D. C., Bayazit, A. K., Canpolat, N., Duzova, A., Sozeri, B., Bacchetta, J., Balat, A., Buscher, A., Candan, C., Cakar, N., Donmez, O., Dusek, J., Heckel, M., Klaus, G., Mir, S., Ozcelik, G., Sever, L., Shroff, R., Vidal, E., Wuhl, E., Gondan, M., Melk, A., Querfeld, U., Haffner, D., Schaefer, F., and Consortium, C. S. (2015) Markers of bone metabolism are affected by renal function and growth hormone therapy in children with chronic kidney disease. *PloS one* **10**, e0113482

27. van der Velde, M., Matsushita, K., Coresh, J., Astor, B. C., Woodward, M., Levey, A., de Jong, P., Gansevoort, R. T., Chronic Kidney Disease Prognosis, C., van der Velde, M., Matsushita, K., Coresh, J., Astor, B. C., Woodward, M., Levey, A. S., de Jong, P. E., Gansevoort, R. T., Levey, A., El-Nahas, M., Eckardt, K. U., Kasiske, B. L., Ninomiya, T., Chalmers, J., Macmahon, S., Tonelli, M., Hemmelgarn, B., Sacks, F., Curhan, G., Collins, A. J., Li, S., Chen, S. C., Hawaii Cohort, K. P., Lee, B. J., Ishani, A., Neaton, J., Svendsen, K., Mann, J. F., Yusuf, S., Teo, K. K., Gao, P., Nelson, R. G., Knowler, W. C., Bilo, H. J., Joosten, H., Kleefstra, N., Groenier, K. H., Auguste, P., Veldhuis, K., Wang, Y., Camarata, L., Thomas, B., and Manley, T. (2011) Lower estimated glomerular filtration rate and higher albuminuria are associated with all-cause and cardiovascular mortality. A collaborative meta-analysis of high-risk population cohorts. *Kidney Int* **79**, 1341-1352
28. Hayek, S. S., Sever, S., Ko, Y. A., Trachtman, H., Awad, M., Wadhwani, S., Altintas, M. M., Wei, C., Hotton, A. L., French, A. L., Sperling, L. S., Lerakis, S., Quyyumi, A. A., and Reiser, J. (2015) Soluble Urokinase Receptor and Chronic Kidney Disease. *The New England journal of medicine* **373**, 1916-1925
29. Cheloha, R. W., Gellman, S. H., Vilardaga, J. P., and Gardella, T. J. (2015) PTH receptor-1 signalling-mechanistic insights and therapeutic prospects. *Nat Rev Endocrinol* **11**, 712-724
30. Zull, J. E., Smith, S. K., and Wiltshire, R. (1990) Effect of methionine oxidation and deletion of amino-terminal residues on the conformation of parathyroid hormone. Circular dichroism studies. *The Journal of biological chemistry* **265**, 5671-5676
31. Frelinger, A. L., 3rd, and Zull, J. E. (1986) The role of the methionine residues in the structure and function of parathyroid hormone. *Arch Biochem Biophys* **244**, 641-649
32. Lopez, I., Rodriguez-Ortiz, M. E., Almaden, Y., Guerrero, F., de Oca, A. M., Pineda, C., Shalhoub, V., Rodriguez, M., and Aguilera-Tejero, E. (2011) Direct and indirect effects of parathyroid hormone on circulating levels of fibroblast growth factor 23 in vivo. *Kidney Int* **80**, 475-482
33. Liu, S. G., Tang, W., Zhou, J. P., Stubbs, J. R., Luo, Q., Pi, M., and Quarles, L. D. (2006) Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. *J Am Soc Nephrol* **17**, 1305-1315
34. Saji, F., Shiizaki, K., Shimada, S., Okada, T., Kunimoto, K., Sakaguchi, T., Hatamura, I., and Shigematsu, T. (2009) Regulation of Fibroblast Growth Factor 23 Production in Bone in Uremic Rats. *Nephron Physiol* **111**, 59-66
35. Samadfam, R., Richard, C., Nguyen-Yamamoto, L., Bolivar, I., and Goltzman, D. (2009) Bone Formation Regulates Circulating Concentrations of Fibroblast Growth Factor 23. *Endocrinology* **150**, 4835-4845
36. Burnett-Bowie, S. A. M., Henao, M. P., Dere, M. E., Lee, H., and Leder, B. Z. (2009) Effects of hPTH(1-34) Infusion on Circulating Serum Phosphate, 1,25-Dihydroxyvitamin D, and FGF23 Levels in Healthy Men. *J Bone Miner Res* **24**, 1681-1685
37. Gutierrez, O. M., Smith, K. T., Barchi-Chung, A., Patel, N. M., Isakova, T., and Wolf, M. (2012) (1-34) Parathyroid Hormone Infusion Acutely Lowers Fibroblast Growth Factor 23 Concentrations in Adult Volunteers. *Clin J Am Soc Nephro* **7**, 139-145
38. Wolf, M. (2010) Forging Forward with 10 Burning Questions on FGF23 in Kidney Disease. *J Am Soc Nephrol* **21**, 1427-1435
39. Xu, G., Luo, K., Liu, H., Huang, T., Fang, X., and Tu, W. (2015) The progress of inflammation and oxidative stress in patients with chronic kidney disease. *Ren Fail* **37**, 45-49

40. Lang, F., Leibrock, C., Pandyra, A. A., Stournaras, C., Wagner, C. A., and Foller, M. (2018) Phosphate Homeostasis, Inflammation and the Regulation of FGF-23. *Kidney & blood pressure research* **43**, 1742-1748
41. Ursem, S. R., Francic, V., Keppel, M., Schwetz, V., Trummer, C., Pandis, M., Aberer, F., Grubler, M., Verheyen, N. D., Maerz, W., Tomaschitz, A., Pilz, S., Obermayer-Pietsch, B. R., and Heijboer, A. C. (2019) The effect of vitamin D supplementation on plasma non-oxidised PTH in a randomised clinical trial. *Endocrine connections*
42. Hocher, B., and Zeng, S. F. (2018) Need for better PTH assays for clinical research and patient treatment. *Clinical chemistry and laboratory medicine* **56**, 183-185
43. Lu, Y. P., Zeng, S., Chu, C., Hasan, A. A., Slowinski, T., Yin, L. H., Kramer, B. K., and Hocher, B. (2020) Non-oxidized PTH (n-oxPTH) is associated with graft loss in kidney transplant recipients. *Clin Chim Acta* **508**, 92-97
44. Cavalier, E., Lukas, P., Warling, X., Moonen, M., Smelten, N., and Delanaye, P. (2020) The percentage of non-oxidized PTH concentration remains stable over a period of 1 year in hemodialyzed patients. *Clin Chim Acta* **506**, 107-109
45. Ursem, S. R., Vervloet, M. G., de Jongh, R. T., and Heijboer, A. C. (2020) Oxidation of parathyroid hormone. *Clin Chim Acta* **506**, 84-91
46. Ursem, S., Francic, V., Keppel, M., Schwetz, V., Trummer, C., Pandis, M., Aberer, F., Grubler, M. R., Verheyen, N. D., Marz, W., Tomaschitz, A., Pilz, S., Obermayer-Pietsch, B., and Heijboer, A. C. (2019) The effect of vitamin D supplementation on plasma non-oxidised PTH in a randomised clinical trial. *Endocr Connect* **8**, 518-527
47. Tepel, M., Armbruster, F. P., Gron, H. J., Scholze, A., Reichetzeder, C., Roth, H. J., and Hocher, B. (2013) Nonoxidized, biologically active parathyroid hormone determines mortality in hemodialysis patients. *J Clin Endocrinol Metab* **98**, 4744-4751

7. Statutory Declaration

“I, Shufei Zeng, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic **“Non-oxidized PTH - but not oxidized PTH - stimulates FGF23 and increases only moderately in CKD patients”, “Im Gegensatz zu oxidiertem PTH zeigt nicht oxidiertes PTH nur einen moderaten Anstieg in CKD Patienten und ist in der Lage Fgf23 zu stimulieren”**, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

8. Declaration of individual contribution to the following publication:

Zeng S, Querfeld U, Feger M, Haffner D, Hasan AA, Chu C, Slowinski T, Bernd Dschietzig T, Schäfer F, Xiong Y, Zhang B, Rausch S, Horvathova K, Lang F, Karl Krämer B, Föller M, Hocher B. Relationship between GFR, intact PTH, oxidized PTH, non-oxidized PTH as well as FGF23 in patients with CKD. FASEB J. 2020 Nov;34(11):15269-15281. doi: 10.1096/fj.202000596R (IF= 4.966)

Contribution in detail:

The aim of this publication was to explore the relationship of PTH and its oxidative derivates with FGF23. I participated in the development of the concept and conducted all the data analysis of the cell experiment and the 4C study cohort. I manually created the figures of FGF23 expression in cell culture (Fig.1), the correlation of n-oxPTH and FGF23 (Fig.2) and analyte concentration according to increments of eGFR (Fig.3). In addition, I designed the table 1&2 to analyze the demographic of two cohorts and table 3&4 showed correlations between n-oxPTH, oxPTH and C-terminal FGF23. Furthermore, I also designed the table 5 and table 6 to show the correlation of iPTH, n-oxPTH, oxPTH, and several CKD-MBD biomarkers. Based on of all data of this study, I create a graphical abstract of the main findings of the present study (Fig.4). In addition, I conducted all supplementary information, including Table S1 to S5 and Figure S1 to S3 in publication. Finally, I wrote the original draft and I did all the new analysis based on reviewer comments and suggestions and revised the final draft.

_____

Signature, date and stamp of first supervising university professor / lecturer

_____

Signature of doctoral candidate

9. Journal Data Filtered By: Selected JCR Year: 2018 Selected Editions: SCIE, SSCI Selected Categories: “BIOLOGY” Selected Category Scheme: WoS

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **“BIOLOGY”** Selected Category Scheme: WoS
Gesamtanzahl: 87 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	Physics of Life Reviews	1,514	11.045	0.002650
2	BIOLOGICAL REVIEWS	12,482	10.288	0.019280
3	PLOS BIOLOGY	29,974	8.386	0.057950
4	eLife	37,014	7.551	0.259540
5	BMC BIOLOGY	5,937	6.723	0.017800
6	BIOSCIENCE	18,353	6.591	0.012840
7	PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES	45,388	6.139	0.071070
8	FASEB JOURNAL	41,854	5.391	0.047670
9	BIOELECTROCHEMISTRY	4,476	4.474	0.004050
10	BIOESSAYS	9,911	4.396	0.016740
11	PROCEEDINGS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES	53,240	4.304	0.079660
12	Geobiology	2,204	4.100	0.004460
13	Current Opinion in Insect Science	1,563	3.784	0.006370
14	ASTROBIOLOGY	3,625	3.768	0.005340
15	Science China-Life Sciences	2,451	3.583	0.006090
16	QUARTERLY REVIEW OF BIOLOGY	4,146	3.519	0.001280
17	Biology Letters	9,783	3.323	0.019530
18	Interface Focus	1,942	3.092	0.004850
19	JOURNAL OF EXPERIMENTAL BIOLOGY	32,933	3.017	0.033230
20	Biology Direct	1,864	3.010	0.003690
21	SAUDI JOURNAL OF BIOLOGICAL SCIENCES	2,746	2.820	0.004410
22	RADIATION RESEARCH	8,561	2.779	0.006480
23	CHRONOBIOLOGY INTERNATIONAL	5,042	2.562	0.006880
24	BIOLOGICAL RESEARCH	1,472	2.482	0.001910
25	JOURNAL OF BIOLOGICAL RHYTHMS	3,069	2.473	0.003570
26	Journal of Biological Research-Thessaloniki	330	2.364	0.000600
27	COMPUTERS IN BIOLOGY AND MEDICINE	5,694	2.286	0.008580
28	INTERNATIONAL JOURNAL OF RADIATION BIOLOGY	4,537	2.266	0.003740
29	CRYOBIOLOGY	4,553	2.141	0.004040
30	Advances in Experimental Medicine and Biology	19,627	2.126	0.033870
31	EXCLI Journal	1,307	2.112	0.002750

10. Manuscript of the publication

Received: 17 March 2020 | Revised: 7 August 2020 | Accepted: 8 September 2020

DOI: 10.1096/fj.202000596R

RESEARCH ARTICLE

THE FASEB JOURNAL

Relationship between GFR, intact PTH, oxidized PTH, non-oxidized PTH as well as FGF23 in patients with CKD

Shufei Zeng[1,2] | **Uwe Querfeld**[3] | **Martina Feger**[4] | **Dieter Haffner**[5] | **Ahmed A. Hasan**[1,6] | **Chang Chu**[1,2] | **Torsten Slowinski**[2] | **Thomas Bernd Dschietzig**[7] | **Franz Schäfer**[8] | **Yingquan Xiong**[1,6] | **Bingbing Zhang**[9,10] | **Steffen Rausch**[4] | **Katarina Horvathova**[11] | **Florian Lang**[6] | **Bernhard Karl Krämer**[1] | **Michael Föller**[4] | **Berthold Hocher**[1,12,13,14]

[1]Fifth Department of Medicine (Nephrology/Endocrinology/Rheumatology), University Medical Centre Mannheim, University of Heidelberg, Heidelberg, Germany

[2]Department of Nephrology, Charité-Universitätsmedizin Berlin, Berlin, Germany

[3]Division of Gastroenterology, Nephrology and Metabolic Diseases, Department of Pediatrics, Charité-Universitätsmedizin Berlin, Berlin, Germany

[4]Department of Physiology, University of Hohenheim, Stuttgart, Germany

[5]Department of Pediatric Kidney, Liver and Metabolic Diseases, Pediatric Research Center, Hannover Medical School, Hannover, Germany

[6]Institute of Nutritional Science, University of Potsdam, Potsdam, Germany

[7]Department of Cardiology, Charité-Universitätsmedizin Berlin, Berlin, Germany

[8]Center for Pediatrics and Adolescent Medicine, University of Heidelberg, Heidelberg, Germany

[9]Institute of Physiology, University of Tübingen, Tübingen, Germany

[10]College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing, China

[11]Biomedica Slovakia s.r.o., Bratislava, Slovakia

[12]Key Laboratory of Study and Discovery of Small Targeted Molecules of Hunan Province, School of Medicine, Hunan Normal University, Changsha, China

[13]Reproductive and Genetic Hospital of CITIC-Xiangya, Changsha, China

[14]Institute of Medical Diagnostics, IMD, Berlin, , Berlin, Germany

Correspondence
Berthold Hocher, Fifth Department of Medicine (Nephrology/Endocrinology/Rheumatology), University Medical Centre Mannheim, University of Heidelberg, Heidelberg, Germany.
Email: berthold.hocher@medma.uni-heidelberg.de

Abstract

Fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH) are regulators of renal phosphate excretion and vitamin D metabolism. In chronic kidney disease (CKD), circulating FGF23 and PTH concentrations progressively increase as renal function declines. Oxidation of PTH at two methionine residues (positions 8 and 18) causes a loss of function. The impact of n-oxPTH and oxPTH on FGF23 synthesis, however, and how n-oxPTH and oxPTH concentrations are affected by CKD, is yet

Abbreviations: 4C, cardiovascular comorbidity in children with chronic kidney disease; BAP, bone alkaline phosphatase; cFGF23, C-terminal FGF23; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; FGF23, fibroblast growth factor 23; GFR, glomerular filtration rate; GPCR, G protein-coupled receptor; HbA1c, hemoglobin A1c; HF, heart failure; iFGF23, intact FGF23; iPTH, intact PTH; Met8(ox)-PTH, PTH oxidized at Met8; Met18(ox)-PTH, PTH oxidized at Met18; Met8, Met18(di-ox)-PTH, PTH oxidized at Met 8 and Met18; n-oxPTH, non-oxidized PTH; Nurr1, nuclear receptor-associated protein-1; oxPTH, oxidized PTH; PTH, parathyroid hormone; PTHR1, parathyroid hormone/parathyroid hormone-related protein receptor 1.

Funding information
China Scholarship Council supported SZ, C.C, and YX This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector

unknown. The effects of oxidized and non-oxidized PTH 1-34 on *Fgf23* gene expression were analyzed in UMR106 osteoblast-like cells. Furthermore, we investigated the relationship between n-oxPTH and oxPTH, respectively, with FGF23 in two independent patients' cohorts (620 children with CKD and 600 kidney transplant recipients). While n-oxPTH stimulated *Fgf23* mRNA synthesis in vitro, oxidation of PTH in particular at Met8 led to a markedly weaker stimulation of *Fgf23*. The effect was even stronger when both Met8 and Met18 were oxidized. In both clinical cohorts, n-oxPTH—but not oxPTH—was significantly associated with FGF23 concentrations, independent of known confounding factors. Moreover, with progressive deterioration of kidney function, intact PTH (iPTH) and oxPTH increased substantially, whereas n-oxPTH increased only moderately. In conclusion, n-oxPTH, but not oxPTH, stimulates *Fgf23* gene expression. The increase in PTH with decreasing GFR is mainly due to an increase in oxPTH in more advanced stages of CKD.

KEYWORDS
CKD, FGF23, GFR, non-oxidized, oxidized, PTH

1 | INTRODUCTION

Fibroblast growth factor 23 (FGF23) is synthesized by bone cells and regulates phosphate homeostasis and vitamin D metabolism.[1] In the kidney, FGF23 inhibits phosphate reabsorption by stimulating the internalization of phosphate transporter NaPiIIa and suppresses the formation of $1,25(OH)_2D_3$ (calcitriol), the active form of vitamin D, by inhibiting 25-hydroxyvitamin D_3 1-alpha-hydroxylase.1 These effects result in a decrease of plasma phosphate and calcitriol concentration. In the parathyroid gland, FGF23 suppresses the formation of parathyroid hormone (PTH),[2] another regulator of phosphate homeostasis, vitamin D, and calcium metabolism. In patients with chronic kidney disease (CKD), FGF23 is a powerful predictor of mortality.[3]

In CKD, an elevation of FGF23 plasma concentrations occurs very early in the disease, even before the onset of appreciable hyperparathyroidism (as measured by currently used intact PTH assays) and hyperphosphatemia, which emerge with progressive decrease in the glomerular filtration rate (GFR).[4,5]

Known regulators of FGF23 production include PTH,[6] $1,25(OH)_2D_3$,[7] the iron status,[8] calcium,[9] dietary phosphate,[10] inflammation,[8] or TGFβ2.[11] In most previous studies, PTH (measured with conventional iPTH assays) and FGF23 serum levels were found to be tightly correlated. It is believed that FGF23 and PTH mutually regulate each other in a negative feedback loop, where PTH stimulates FGF23 production and FGF23 in turn suppresses PTH synthesis.[6] Meir et al[12] demonstrated that PTH can activate the nuclear receptor-associated protein-1 (Nurr1) via the PTH receptor to induce *FGF23* transcription in bone cells.

PTH 1-84 has two methionine amino acids at positions 8 and 18, respectively. They are located within the receptor binding site. These methionine amino acids can be oxidized in vivo,[13-15] resulting in diminished binding to the PTH receptor.

Several studies clearly indicate that the PTH oxidation is critical for the biological activity of PTH. We have previously described the presence of a variable amount of oxPTH in the circulation of patients with CKD, on dialysis and after kidney transplantation.[13] The relationship between oxPTH and n-oxPTH is highly variable and at present cannot be predicted in individual patients.[13-15]

Here, we explored the relationship of PTH and its oxidative derivates with FGF23. We analyzed the effects of oxPTH and n-oxPTH 1-34 on *Fgf23* gene expression in UMR106 osteoblast-like cells. Furthermore, we investigated the relationship between n-oxPTH, oxPTH, and iPTH (the sum of n-oxPTH and oxPTH) with FGF23 in two independent patient cohorts (620 children with CKD and 600 kidney transplant recipients) considering known confounding factors[6,9,10,16] and likewise analyzed the relationship between GFR and iPTH, oxPTH, n-oxPTH as well as FGF23.

2 | MATERIALS AND METHODS

2.1 | Patients

We analyzed plasma samples of children participating in the Cardiovascular Comorbidity in Children with CKD (4C) Study, a prospective observational cohort study in children aged from 6 to 17 years with a GFR of 10 to 60 mL/

min/1.73 m^2 at study entry.[17] The study was approved by the Ethics Commission of the University of Heidelberg. The patient information and consent forms (translated into the national language) were reviewed and approved by the local Institutional Review Boards in each participating institution. At start of the study and during follow-up visits every 6 months, blood and urine samples were collected, stored, and analyzed in a central laboratory. Demographic data for children (age, gender, height, BMI etc) were extracted from hospital records of the patients. The estimated glomerular filtration rate (eGFR) was calculated using a previously published cystatin C- and creatinine-based equation.[18]

We also analyzed plasma samples from a kidney transplant patient cohort, for details, see 13. Six-hundred adult kidney transplant recipients with a functioning graft were enrolled in this prospective study, which was conducted at the transplant clinic Charité-Mitte, Berlin, Germany. Patients were excluded if they had any acute infection, malignancy, acute rejection, acute myocardial infarction, pulmonary edema, or heart failure (HF) at the time of blood sampling. The eGFR was calculated according to CKD-EPI equations.

We divided patients of each cohort into seven groups (for details, see, Figure 3) according to eGFR and calculated the mean (SEM) circulating concentration of each analyte in patients within different eGFR intervals to observe trends in analytes concentrations and the relationship between each other as eGFR decreased.

2.2 | Laboratory methods

Albumin, creatinine, cholesterol, HbA1c, $1,25(OH)_2D_3$, calcium, phosphate, fasting blood glucose, and urinary protein were measured by standardized laboratory techniques in both cohorts.

The intact-PTH electrochemiluminescence immunoassay (ECLIA; Roche PTH, Intact [iPTH]) uses a biotinylated monoclonal antibody, which reacts with amino acids 26-32, and a capture ruthenium-complexed monoclonal antibody, which reacts with amino acids 55-64. The determinations were performed on Roche Modular E 170. The currently in clinical practice used second and third generation PTH assays (also called intact PTH assays) cannot discriminate between oxPTH and n-oxPTH. They measure both together: oxPTH and n-oxPTH (iPTH = n-oxPTH + oxPTH). Samples were measured either directly (named iPTH) or after removal of oxPTH by a column using anti-human-oxPTH monoclonal antibodies as described in detail elsewhere.[15]

ELISA kits were also used for quantitative determination of FGF23. We used the following ELISAs: The cFGF23 ELISA (C-terminal) multi-matrix ELISA, BI-20702 (Biomedica, Austria; https://www.bmgrp.com/wp-content/uploads/2019/03/bi-20702-fgf23-elisa-validation-data-150306 .pdf), and iFGF23 (intact) human ELISA, BI-20700 (Biomedica, Austria, see; https://www.bmgrp.com/wp-content/uploads/2019/06/BI-20700-FGF23-Intact-ELISA-Validation-Data-CE-190808.pdf) were used for the transplant study. The cFGF-23 ELISA kit from Immutopics (Immutopics, San Clemente, CA, USA; verification and validation, see: https://www.quidel.com/sites/default/files/product/documents/PI6061001EN00.pdf) was used in the pediatric 4C study (Table S1). The technical details of ELISAs used for the measurement of sclerostin, osteoprotegerin (OPG), tartrate-resistant acid phosphatase 5b (TRAP5b), oxidized LDL (oxLDL), carbonyl proteins, and advanced oxidation protein products (AOPP) are given in the supplementary materials.

All samples were measured in duplicate and all assays were subject to regular quality control.

2.3 | Cell culture

UMR106 rat osteoblast-like cells (ATCC, CRL-1661) were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 10% FBS (Gibco, Life Technologies) and 100 U/mL penicillin/100 µg/mL streptomycin (Gibco, Life Technologies) under standard culture conditions as described by Bär et al recently.19 Cells were first grown for 24 hours and then treated with or without the different PTH 1-34 derivatives as indicated for additional 24 hours. Non-oxidized PTH (n-oxPTH), PTH oxidized at Met8 (Met8(ox)-PTH), PTH oxidized at Met18 (Met18(ox)-PTH) as well as PTH oxidized at both Methionine amino acids (Met 8 and Met18) (Met8, Met18(di-ox)-PTH) were obtained from JPT Peptide Technologies GmbH—Volmerstraße 5 (UTZ)—12489 Berlin, Germany.

HPLC analysis demonstrated the purity of >95% of all peptides (data not shown). We have recently reported[15] that the affinity column completely removes oxPTH as controlled by LC-MS. Additional stability experiments showed that n-oxPTH is independent of the time on column (between 10 and 120 minutes). The proportion of oxPTH remaining on the column is substantially different between CKD patients and healthy controls indicating that the vast majority of PTH is oxidized in CKD patients but not in healthy controls, see, Figure S1. Relative transcript levels were quantified by qRT-PCR: RNA was extracted with peqGOLD TriFast (Peqlab, Erlangen, Germany), cDNA synthesized using GoScript Reverse Transcription System and random primers (both from Promega, Mannheim, Germany), followed by qRT-PCR with GoTaq qPCR Master Mix (Promega) (95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, 57°C

(*Fgf23*, *Rpl13a*), 54°C (*Il6*), 55°C (*G6pd*), 58°C (*Actb*), or 60°C (*Sost*) for 30 seconds, and 72°C for 25 seconds) using following rat primers:

Fgf23: 5′-TAGAGCCTATTCAGACACTTC and 5′-CATCAGGGCACTGTAGATAG; *Rpl13a*: 5′-GCACAAGACCAAAAGAGG and 5′-CGCTTTTTCTTGTCATAGGG; *Actb*: 5′-CGCCACCAGTTCGCCAT and 5′-TACCCACCATCACACCCTGG; *G6pd*: 5′-ATTGCTTGTGTGATCCTTAC and 5′-CACTTTGACCTTCTCATCAC; *Il6*: 5′-CAGAGTCATTCAGAGCAATAC and 5′-CTTTCAAGATGAGTTGGATGG; *Sost*: 5′-ATGATGCCACAGAAATCATC and 5′-CACGTCTTTGGTGTCATAAG.

2.4 | Statistics

Relative gene expression of *Fgf23*, *Actb*, *G6pd*, *Il6*, and *Sost* in cultured UMR106 cells treated with n-oxPTH- and different forms of ox-PTH-peptides were compared by one-way ANOVA followed by Dunnett's multiplecomparisons test.

Analysis of the statistical difference in the mean value of each parameter of the three patient groups (CKD stages 1-2, stage 3, and stages 4-5) of both clinical CKD cohorts was performed by one-way ANOVA. The values of the variables with skewed distribution were ln transformed. Linear multivariate regression models considering known confounding factors for FGF23 syntheses were used to investigate the correlation between FGF23 and either oxPTH or n-oxPTH in the clinical studies. All analyses were performed using SPSS, version 19 and differences were considered significant if $P < .05$.

3 | RESULTS

3.1 | In vitro studies

To determine the effect of PTH oxidation on the regulation of *Fgf23* transcription, UMR106 osteoblast-like cells were treated with n-oxPTH- and oxPTH-derivatives. The quality of the used PTH peptides was proven by HPLC (quality reports shown in the supplements). As shown in Figure 1, we performed a dose response curve investigating the following concentrations of the four PTH peptides in the cell culture medium: 3, 10, 30, 100, and 300 nmol/L. All dosages of n-oxPTH led to a stimulation of *Fgf23* mRNA synthesis. Neither Met8, Met18 di-oxPTH nor Met8 oxPTH caused a significant stimulation of *Fgf23* mRNA expression at any of the investigated dosages. Met18 oxPTH has a partial agonistic effect on *Fgf23* mRNA synthesis, see, Figure 1. Beside the stimulation of FGF23 by n-oxPTH and different forms of oxPTH in UMR106 cells, we also did the experiments with positive (interleukin-6 (*Il6*) and sclerostin (*Sost*),[20-26]) and negative (actin (*Actb*) and glucose-6-phosphate-dehydrogenase (*G6pd*)) controls, see, Figure S2.

3.2 | Patients cohorts

Demographics and characteristics of the 4C study population are presented in Table 1. A total of 620 children with CKD, 404 males (65.2%) and 216 females (34.8%) were included in this study. Twenty-one patients were found to have a higher than required GFR (>60 mL/min/1.73 m^2) after

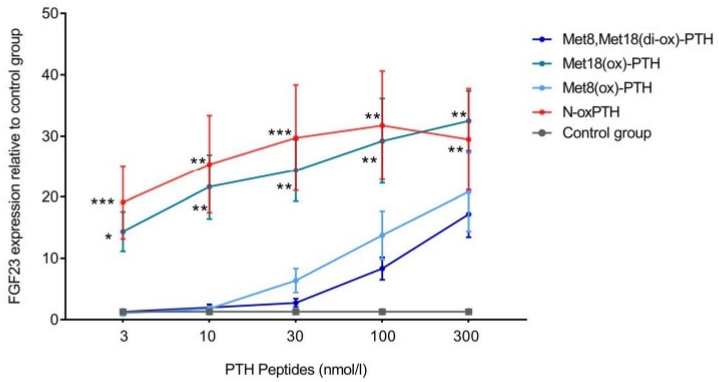


FIGURE 1 Effect of non-oxidized PTH (n-oxPTH) and different forms of oxidized PTH on *Fgf23* expression in cultured UMR106 osteoblast-like cells. Cells were first grown for 24 hours and then, treated with or without the addition of various dosages (3, 10, 30, 100, and 300 nmol/L) of the different PTH 1-34 derivatives as indicated for additional 24 hours. The experiment was repeated six to seven times and *Fgf23* gene expression (relative to *Rpl13a*) was measured by qRT-PCR. All dosages of n-oxPTH and Met18 oxPTH led to a significant stimulation of *Fgf23* mRNA synthesis (Dunnett's test). Neither Met8, Met18 di-oxPTH nor Met8 oxPTH caused a significant stimulation of *Fgf23* mRNA expression at any of the investigated dosages. The x axis denotes the concentration. The y axis denotes relative *Fgf23* expression.

TABLE 1 Patient characteristics of children with CKD (n = 620)

	All	Stages 1-2	Stage 3	Stages 4-5	*P*
N	620	21	296	298	
Sex (female/male)	216 f/404 m	7 f/14 m	93 f/203 m	114 f/184 m	
Age at study entry (years)	12.1 (3.3)	11.7 (2.9)	12.2 (3.3)	12.1 (3.4)	.691
Height standard deviation score	−1.4 (1.5)	−1.1 (1.7)	−1.1 (1.5)	−1.6 (1.4)	<.001
Cystatin C (mg/L)	2.8 (0.9)	1.4 (0.3)	2.3 (0.4)	3.5 (0.8)	<.001
eGFR (mL/min/1.73 m^2)	32.8 (13.3)	72.6 (14.3)	40.3 (7.5)	22.5 (4.6)	<.001
Plasma calcium(mmol/L)	2.2 (0.2)	2.2 (0.3)	2.2 (0.2)	2.2 (0.2)	.839
Plasma phosphate (mmol/L)	1.5 (0.4)	1.5 (0.6)	1.4 (0.4)	1.6 (0.4)	<.001
Sclerostin (ng/mL)	0.32 (0.14)	0.31 (0.14)	0.31 (0.16)	0.33 (0.15)	.225
TRAP5b (U/L)	12.7 (6.5)	13.0 (6.2)	11.7 (5.9)	12.6 (6.9)	.587
BAP (mcg/L)	150.0 (84.8)	134.3 (78.0)	146.5 (77.1)	155.3 (92.5)	.315
Plasma creatinine (mg/dL)	2.6 (1.4)	0.9 (0.2)	1.8 (0.5)	3.6 (1.4)	<.001
Intact PTH (pg/mL)	163.5 (172.3)	59.9 (44.9)	123.5 (126.7)	211.5 (202.4)	<.001
N-oxPTH (pg/mL)	17.4 (14.6)	13.8 (23.2)	13.6 (11.2)	21.5 (15.8)	<.001
OxPTH (pg/mL)	146.5 (160.8)	46.1 (51.3)	110.4 (118.0)	190.0 (188.9)	<.001
C-terminal FGF23 (pg/mL)	322.4 (491.6)		204.6 (321.5)	444.4 (598.2)	<.001

Note: Numbers are presented as mean (SD) or n. The 4C study provides cFGF23 data only for patients with a GFR < 60 mL/min/1.73 m^2 without reason. Analysis of the statistical difference in the mean value of each parameter of the three patient groups (CKD stages 1-2, stage 3, and stages 4-5) using one-way ANOVA. Two-sided independent T test was used for analysis of the statistical difference in the mean value of C-terminal FGF23 in CKD stage 3 and stages 4-5. There are five patients without GFR data. Patients were classified according to CKD stages as described.[47,48]

Abbreviations: BAP, bone alkaline phosphatase; TRAP5b, tartrate-resistant acid phosphatase 5b.

enrollment than at the screening visit, but were kept in the study (stages 1-2 CKD). At study entry, mean age of patients was 12.1(3.3) years, and mean eGFR was 32.8(13.3) mL/min/1.73 m^2. The mean (SD) baseline height standard deviation score was −1.4(1.5). The mean plasma concentrations of iPTH, n-oxPTH, and oxPTH were 163.5(172.3) pg/mL (n = 620), 17.4(14.6) pg/mL (n = 618), and 146.5 (160.8) pg/mL (n = 618), respectively. We used cFGF23 data[27] of the 4C study, which had been measured in patients with a GFR of less than 60 mL/min/1.73 m^2 (n = 552); the mean cFGF23 concentration was 322.4(491.6) pg/mL.

Patient demographics and relevant clinical and biochemical parameters for kidney transplant recipients are summarized in Table 2. A total of 600 stable renal transplant recipients aged 20 to 87 years, were included in this study. At study entry, mean age of patients was 54.6 (14.5) years, and the mean eGFR was 44.4 (18.0) mL/min/1.73 m^2. The mean cFGF23 concentration was 103.6 (1112.5) pg/mL (n = 556). The bone alkaline phosphatase (BAP) activity was correlated with the n-oxPTH/oxPTH ratio in both cohorts (CKD study in children: Spearman's rho: 0.207, $P < .001$; transplant cohort: Spearman's rho: 0.201, $P < .001$, respectively), but not with oxPTH ($P > .05$ in both studies).

In order to translate the cell culture findings showing that n-oxPTH stimulates the expression of *Fgf23* in vitro to clinical science, we investigated the relationship of n-oxPTH, oxPTH, and FGF23 in two independent CKD cohorts: children with CKD and kidney transplant recipients. We observed a significant positive correlation between the cFGF23 concentration and n-oxPTH (Figure 2, $P < .001$).

Since FGF23 secretion is influenced also by other factors, we considered the following confounding factors in a multivariate regression analysis: patient age, GFR stage, calcium, phosphate, height standard deviation score (only used in

TABLE 2 Patient characteristics of adult kidney transplant recipients (n = 600)

	All	Stages 1-2	Stage 3	Stages 4-5	*P*
N	600	114	336	126	
Sex (female/male)	233 f/367 m	44 f/70 m	126 f/210 m	57 f/69 m	
Age at study entry (years)	54.6 (14.5)	50.0 (13.9)	54.6 (14.5)	59.0 (13.8)	<.001
Donor age (years)	49.4 (15.8)	38.9 (14.3)	49.8 (15.0)	57.0 (14.7)	<.001
Time on dialysis (months)	49.5 (35.2)	47.2 (38.3)	48.6 (35.6)	55.0 (31.8)	.199
Time post-transplantation (months)	89.9 (75.3)	99.8 (79.3)	87.7 (72.3)	91.5 (77.7)	.333
Cold ischemia time (hours)	10.1 (7.3)	9.6 (8.3)	9.8 (7.0)	11.7 (7.1)	.035
eGFR (mL/min/1.73 m^2)	44.4 (18.0)	71.2 (11.2)	43.6 (8.8)	22.3 (4.7)	<.001
Plasma albumin (g/dL)	4.6 (0.4)	4.6 (0.3)	4.6 (0.4)	4.4 (0.3)	.003
Plasma creatinine (mg/dL)	1.8 (0.7)	1.1 (0.2)	1.6 (0.3)	2.8 (0.7)	<.001
Total cholesterol (mg/dL)	223.1 (53.0)	211.2 (49.1)	221.3 (49.5)	236.6 (60.4)	.001
HbA1c (%)	5.9 (0.9)	5.8 (0.7)	5.9 (0.9)	5.9 (0.9)	.330
Plasma $1,25(OH)_2D$ (pmol/L)	96.2 (54.4)	114.5 (53.4)	100.7 (53.5)	69.5 (48.7)	<.001
Plasma calcium (mmol/L)	2.5 (0.2)	2.5 (0.2)	2.5 (0.2)	2.5 (0.2)	.325
Plasma phosphate (mmol/L)	0.9 (0.3)	0.8 (0.2)	0.9 (0.2)	1.1 (0.3)	<.001
Sclerostin (pmol/l)	48.6 (25.7)	60.1 (31.2)	47.1 (22.5)	41.0 (24.9)	<.001
OPG (pmol/l)	4.9 (2.5)	6.3 (3.3)	4.7 (2.3)	3.9 (1.4)	<.001
BAP (mcg/L)	20.3 (11.1)	21.7 (14.5)	20.0 (10.1)	20.0 (10.2)	.350
Fasting blood glucose (mg/dL)	95.6 (35.1)	89.0 (26.3)	96.4 (34.7)	98.2 (42.7)	.089
Urinary protein (mg/24 h)	366.3 (646.6)	216.3 (449.5)	293.3 (456.8)	709.4 (1025.8)	<.001
Intact PTH (pg/mL)	113.9 (129.5)	72.2 (59.0)	102.8 (78.5)	172.4 (227.4)	<.001
N-oxPTH (pg/mL)	12.0 (12.6)	8.0 (5.6)	10.7 (7.9)	18.0 (21.5)	<.001
OxPTH (pg/mL)	101.9 (117.9)	64.2 (54.0)	92.2 (72.1)	154.4 (206.5)	<.001
C-terminal FGF23 (pg/mL)	103.6 (1112.5)	12.5 (20.6)	21.5 (40.3)	344.9 (2315.3)	.016

Note: Numbers are presented as mean (SD) or n. Analysis of the statistical difference in the mean value of each parameter of the three patient groups (CKD stages 1-2, stage 3, and stages 4-5) using one-way ANOVA. Patients were classified according to CKD stages as described.[47,48]

Abbreviations: BAP, bone alkaline phosphatase; OPG, osteoprotegerin.

children with CKD), and donor age (only used in kidney transplant recipients). In children with CKD, we observed that the cFGF23 concentration was independently associated with the concentration of n-oxPTH (95% CI 0.011-0.236; $P = .0313$), but not oxPTH (95% CI −0.074-0.076; $P = .9742$) (Table 3). Similar results were observed in kidney transplant recipients; the cFGF23 concentration was independently associated with n-oxPTH concentration (95% CI 0.220-0.507; $P < .001$), but

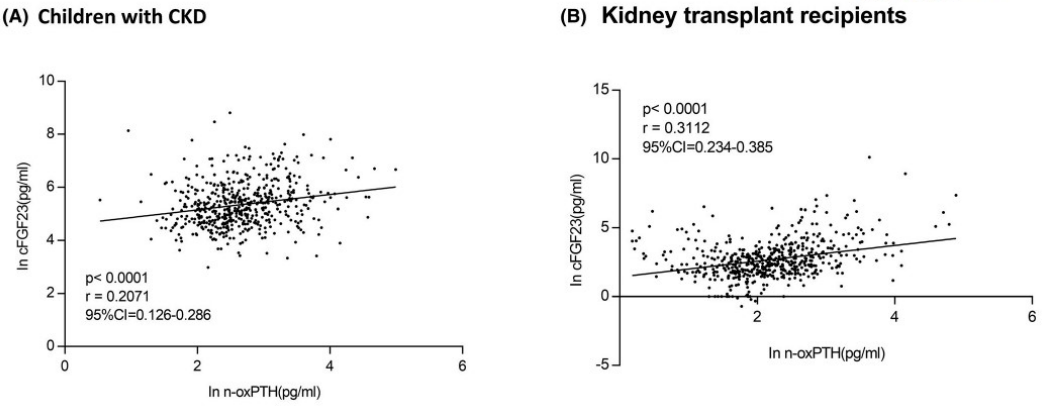


FIGURE 2 N-oxPTH and FGF23 plasma concentrations are positively correlated in children with CKD and in kidney transplant recipients

TABLE 3 Multivariate linear regression of variables associated with C-terminal FGF23 plasma concentrations in children with CKD

	Standardized Beta	*T*	*P*	95% Confidence Interval for *B*
Contains n-oxPTH				
Age	−0.017	−0.437	.662	−0.024-0.015
Height standard deviation score	0.195	5.036	<.001	0.069-0.158
GFR stage	0.384	9.426	<.001	0.470-0.718
Calcium	0.070	1.799	.073	−0.022-0.498
Phosphate	0.169	4.277	<.001	0.211-0.569
N-oxPTH	0.090	2.159	.031	0.011-0.236
Contains oxPTH				
Age	−0.015	−0.380	.704	−0.023-0.016
Height standard deviation score	0.189	4.862	<.001	0.066-0.156
GFR stage	0.409	10.260	<.001	0.512-0.754
Calcium	0.052	1.285	.199	−0.093-0.445
Phosphate	0.184	4.588	<.001	0.242-0.604
OxPTH	0.001	0.032	.974	−0.074-0.076

Note: GFR stage: stage 1: eGFR ≥ 90 mL/min/1.73 m^2; stage 2: eGFR 60-89 mL/min/1.73 m^2; stage 3: eGFR 30-59 mL/min/1.73 m^2; stage 4: eGFR 15-29 mL/min/1.73 m^2; stage 5: eGFR < 15 mL/min/1.73 m^2.

Abbreviations: N-oxPTH, non-oxidized parathyroid hormone; oxPTH, oxidized parathyroid hormone.

not oxPTH (95% CI −0.007-0.192; $P = .069$) (Table 4). Intact PTH—the sum of n-oxPTH and oxPTH—correlated with C-terminal FGF23 in kidney transplant recipients, but not in children with CKD (Tables S2 and S3). When analyzing FGF23 with an intact FGF23 (iFGF23) ELISA, we likewise saw that iFGF23 concentration was independently associated with the concentration of n-oxPTH, but not oxPTH (Table S4).

Taken together, the analysis of the correlation between hormone concentrations (Figure 3) as well as the multivariate linear regression analysis (Tables 3 and 4) indicates that n-oxPTH but not oxPTH is positively and independently of known confounding factors correlated with FGF23 in children with CKD and kidney transplant recipients, respectively.

With decreasing eGFR, iPTH and oxPTH increase substantially at early stages of CKD, whereas n-oxPTH increases moderately at later stages of CKD (Figure 3). Intact PTH increased from 59.9(44.9) pg/mL (CKD stage 1-2) to 211.5(202.4) pg/mL (CKD stages 4-5) in the 4C study population. The increase in iPTH of 151.6 pg/mL in the 4C study is mainly due to an elevation of oxPTH (+143.9 pg/mL), whereas n-oxPTH (+7.7 pg/mL) contributed only to a minor extent to this iPTH increase (Table 1). Comparable findings

TABLE 4 Multivariate linear regression of variables associated with C-terminal FGF23 plasma concentrations in adult kidney transplant recipients

	Standardized Beta	*T*	*P*	95% Confidence Interval for *B*
Contains n-oxPTH				
Age	−0.019	−0.461	.645	−0.009-0.005
GFR stage	0.354	7.619	<.001	0.471-0.798
Calcium	0.095	2.349	.019	0.108-1.218
Phosphate	0.193	4.465	<.001	0.545-1.400
Donor age	−0.056	−1.291	.197	−0.011-0.002
N-oxPTH	0.209	4.982	<.001	0.220-0.507
Contains oxPTH				
Age	−0.005	−0.125	.901	−0.008-0.007
GFR stage	0.398	8.525	<.001	0.549-0.878
Calcium	0.132	3.231	.001	0.361-1.480
Phosphate	0.169	3.764	<.001	0.408-1.298
Donor age	−0.06	−1.361	.174	−0.012-0.002
OxPTH	0.077	1.819	.069	−0.007-0.192

Note: GFR stage: stage 1: eGFR ≥ 90 mL/min/1.73 m^2; stage 2: eGFR 60-89 mL/min/1.73 m^2; stage 3: eGFR 30-59 mL/min/1.73 m^2; stage 4: eGFR 15-29 mL/min/1.73 m^2; stage 5: eGFR < 15 mL/min/1.73 m^2.

Abbreviations: N-oxPTH, non-oxidized parathyroid hormone; oxPTH, oxidized parathyroid hormone.

were obtained in the kidney transplant recipients. Intact PTH increased from 72.2(59.0) pg/mL (CKD stages 1-2) to 172.4(227.4) pg/mL (CKD stages 4-5) in the kidney transplant recipients. The increase in iPTH of 100.2 pg/mL in the transplant study was mainly due to an increase in oxPTH (+90.2 pg/mL), whereas n-oxPTH (+10.0 pg/mL) contributed only to this iPTH increase to a minor extent (Table 2).

We also performed an assessment of other circulating CKD-MBD biomarkers and their relationship with iPTH, n-oxPTH and oxPTH. Sclerostin and OPG was measured in kidney transplant recipients as CKD-MBD biomarker. Sclerostin was correlated with n-oxPTH but not with oxPTH, whereas OPG was correlated with both n-oxPTH and oxPTH. In the 4C study in children, we measured sclerostin and TRAP5b as CKD-MBD markers. Both were correlated with n-oxPTH and oxPTH (see, Tables 5 and 6).

The correlation of biomarkers of lipid-, carbohydrate-, and protein oxidation were analyzed in the initial n-oxPTH outcome study,[28] since we had not enough blood left from the current studies. Biomarkers of lipid and carbohydrate oxidation were not correlated with either ox- or n-oxPTH. Carbonyl proteins as biomarkers of protein oxidation were inversely correlated with n-oxPTH. These data suggest that a higher degree of protein oxidation is linked to lower concentration of n-oxPTH, see, Table S5.

4 | DISCUSSION

This study confirms different biological effects of oxidized and non-oxidized forms of PTH (Figure 4). While n-oxPHT stimulated *Fgf23* mRNA synthesis in vitro, oxidation of PTH particularly at position Met8 led to a markedly weaker stimulation of *Fgf23* mRNA synthesis. In two independent cohorts—children with CKD and adult kidney transplant recipients—only n-oxPTH, but not oxPTH, was associated with FGF23 concentrations, independent of known confounding factors. With progressive deterioration of kidney function, FGF23 concentrations and iPTH increased substantially. However, the biologically active n-oxPTH increased much less than iPTH and oxPTH in both patient cohorts.

The cell culture experiments revealed that n-oxPTH 1-34 stimulates *Fgf23* mRNA synthesis much more potently than PTH 1-34 oxidized particularly at position Met8. These findings are consistent with the literature. The parathyroid hormone/parathyroid hormone-related protein receptor (PTH/PTHrP type 1 receptor also named PTHR1) is a member of the G protein-coupled receptor (GPCR) family that is expressed primarily in bone, kidney, and cartilage but also in other tissues including the vasculature.[29] Ligand structure-activity relationship and receptor mutagenesis studies indicate that bioactive PTH 1-34 interacts with PTHR1 via a two-component mechanism. Thus, the C-terminal portion of PTH 1-34 interacts with the amino-terminal extracellular domain of PTHR1 (site 1), whereas the N-terminal portion interacts with the transmembrane helices and extracellular connecting loops (site 2).[29]

The PTH molecule can be oxidized at methionine residues 8 and 18 and there is overwhelming evidence that oxPTH and n-oxPTH have different biological properties. These findings are reflected in three lines of evidence:

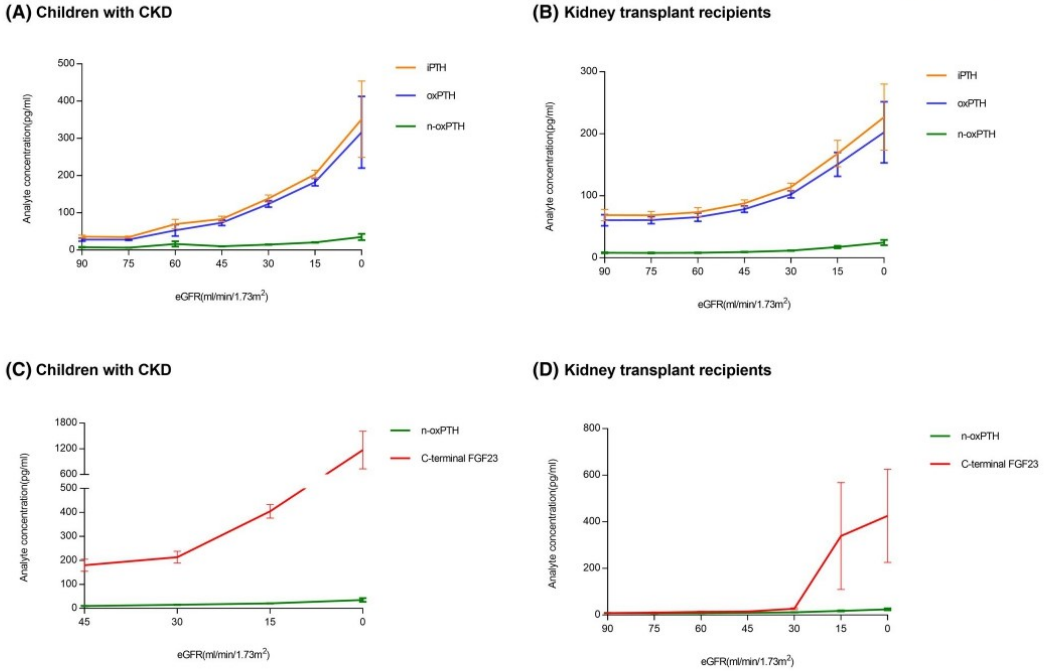


FIGURE 3 Plasma levels of C-terminal FGF23, iPTH, non-oxidized and oxidized forms of PTH according to increments of eGFR in children with CKD and in adult kidney transplant recipients. We divided patients into seven groups according to eGFR and calculated the mean (SEM) circulating concentrations of each analyte in patients with different intervals of eGFR, respectively. 0 represents eGFR > 0 and ≤ 15mL/min/1.73 m^2. 15 represents eGFR > 15 and ≤ 30 mL/min/1.73 m^2. 30 represents eGFR > 30 and ≤ 45 mL/min/1.73 m^2. 45 represents eGFR > 45 and ≤ 60 mL/min/1.73 m^2. 60 represents eGFR > 60 and ≤ 75 mL/min/1.73 m^2. 75 represents eGFR > 75 and ≤ 90 mL/min/1.73 m^2. 90 represents eGFR > 90 mL/min/1.73 m^2. The y axis denotes the mean circulating concentrations of the individual analytes (iPTH [pg/mL] in orange; oxPTH [pg/mL] in blue; n-oxPTH [pg/mL] in green and FGF23 [pg/mL] in red). For the 4C cohort, we used FGF23 data only for patients with a GFR < 60 mL/min/1.73 m^2 as initially reported, see 27. n-oxPTH of children is 4.6 times higher, when eGFR is 0-15 compared to an eGFR > 90 mL/min/1.73 m^2 ($P = .0043$). n-oxPTH in adult kidney transplant recipients is 3.0 times higher, when eGFR is 0-15 compared to an eGFR > 90 mL/min/1.73 m^2 ($P = .0062$).

TABLE 5 Correlation of iPTH, n-oxPTH, oxPTH, and sclerostin, TRAP5b in children with CKD at study entry

		intact PTH	n-oxPTH	oxPTH
Sclerostin	r_s	−0.102	−0.142	−0.141
	P	.014	.001	.001
	n	588	585	590
TRAP5b	r_s	0.192	0.228	0.229
	P	<.001	<.001	<.001
	n	598	595	600

Note: We used Spearman's rho to assess bivariate associations.
Abbreviation: TRAP5b, tartrate-resistant acid phosphatase 5b.

TABLE 6 Correlation of iPTH, n-oxPTH, oxPTH, and sclerostin, OPG in kidney transplant recipients at study entry

		intact PTH	n-oxPTH	oxPTH
Sclerostin	r_s	−0.076	−0.091	−0.069
	P	.070	.030	.100
	n	575	575	574
OPG	r_s	0.134	0.124	0.131
	P	.001	.002	.001
	n	597	596	595

Note: We used Spearman's rho to assess bivariate associations.
Abbreviation: OPG, osteoprotegerin.

a. oxPTH has a much lower binding affinity to the PTH receptor as shown by classic receptor binding assays[13] and oxPTH—in contrast to n-oxPTH—does not stimulate the PTH receptor to generate the second messenger of PTH receptor cAMP.[13]

b. It was shown that oxPTH loses its biologic action on smooth muscle cell contraction/vascular effects in tissues like trachea, aortic rings, and the uterus. Stimulation of alkaline phosphatase activity in cultured neonatal mouse calvarial bone cells by PTH was

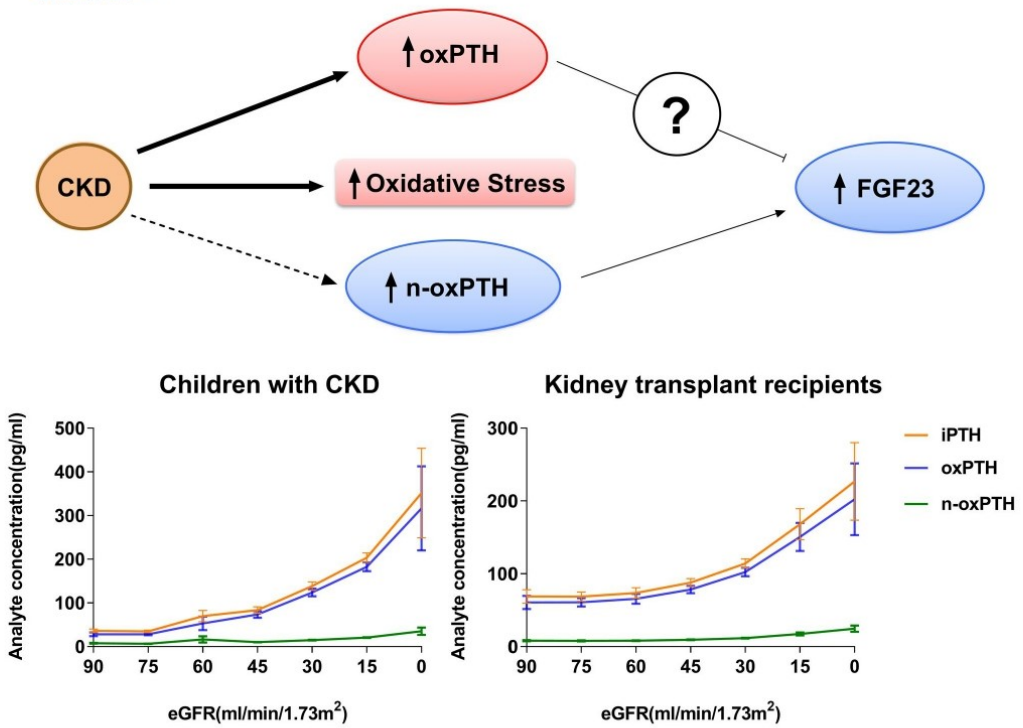


FIGURE 4 A graphical abstract of the main findings of the present study. N-oxPTH but not oxPTH stimulates FGF23 synthesis in vitro. Results from two independent cross-sectional cohort studies are in agreement with this finding showing an independent association of n-oxPTH, but not oxPTH, with FGF23. N-oxPTH increases only moderately with decreasing GFR. The reported increase in PTH in the literature—as measured by iPTH assays—when GFR is decreasing is mainly due to an increase in oxPTH.

seen only after incubation with n-oxPTH but not with oxPTH. Only n-oxPTH (but not oxPTH) is able to regulate calcium and phosphate metabolism in vivo. For more details, see, the review of these studies in Hocher et al.[13]

c. Our finding that the effect of oxidation at methionine 8 seems to be more critical for the effects of PTH on its receptor are in good agreement with structural analysis of oxPTH peptides. This study demonstrated that oxidation of the methionine residue at position 18 has a small impact on the secondary structure, whereas oxidation of the methionine at position 8 produces substantial changes. Oxidation of both methionines produces secondary structure changes that are greater than the sum of those seen upon oxidation of the individual methionines.[30] Another study analyzed the effects of oxidization of either methionine 8 or 18 on the generation of the second messenger cAMP. In line with our data and the data by Zull et al,[30] our data showed that oxidation at methionine 8 was more potent to inhibit the formation of the second messenger of PTH cAMP.[31]

We saw a positive correlation of n-oxPTH—but not oxPTH—with FGF23 in the analyzed cohorts independent of known confounding factors.

Previous in vitro and in vivo studies exploring the relationship of PTH and FGF23 had conflicting results. For example, increases in murine osteocyte FGF23 mRNA expression in vitro via activation of the PTHR1 receptor has been demonstrated,[6,32] whereas some animal studies demonstrated decreased plasma FGF23 concentrations with PTH treatment.[33-35] In humans, Burnett-Bowie and colleagues[36] demonstrated that PTH infusion in healthy men increased FGF23 over 18 hours, while Gutierrez[37] demonstrated acute lowering of FGF23 with PTH infusion within 6 hours. The reason for these conflicting data are not fully understood and might partially be due to differences in the studied populations. We have previously described the presence of high concentrations of circulating ox-PTH in CKD patients[13]; here we show substantial differences compared to n-oxPTH concentrations in their associations with GFR and FGF23, further confirming different biological properties in vivo.

In contrast to the current literature about the relationship between PTH and decreasing GFR,[4,38] the biologically active n-oxPTH increased much less than iPTH and oxPTH with the progressive deterioration of kidney function. These results indicate that the oxidation of PTH progressively increases with decreasing GFR, reflecting the degree of oxidative stress as described by others.[39]

These data suggest that the increase in PTH with decreasing GFR described in textbooks of kidney physiology reflects primarily the elevation of oxPTH but not of n-oxPTH. Our data indicate that the progressive increase in FGF23 with decreasing GFR seems not to be associated with the moderate rise in n-oxPTH, but that other factors might play a more important role in the regulation of FGF-23 levels in CKD.[40]

Several limitations should be taken into consideration when interpreting the results of this study. First, our study suggested that the effect of n-oxPTH on FGF23 is CKD stage-dependent. However, this needs to be confirmed by injecting n-oxPTH in different CKD stage in experimental animal models. Second, the amount of oxidative stress and its impact on circulating concentrations of PTH and FGF23 could not be assessed since markers of oxidative stress were not measured in the clinical studies. In this context, it is important to know that PTH oxidation occurs in vivo and is largely independent of sample handling[14] and seems to be influenced by vitamin D.[41] Lastly, since the assay used for oxPTH in the clinical study cannot distinguish between the different forms of oxPTH, we could not analyze the relationship between each form of oxPTH and FGF23 and their respective trends with the decline of GFR. Clinically suitable assay systems that can differentiate between the PTH forms are needed. Our study may stimulate the development of such methods given the huge clinical impact of PTH.

In conclusion, only n-oxPTH, but not oxPTH, stimulates FGF23 synthesis in vitro. Results from two independent cross-sectional cohort studies are in agreement with this finding showing an independent association of n-oxPTH, but not oxPTH, with FGF23. We could furthermore demonstrate that n-oxPTH increases only moderately with decreasing GFR. The reported increase in PTH in the literature—as measured by iPTH assays—when GFR is decreasing is mainly due to an increase in oxPTH.

We suggest that our findings might be of general importance and go far beyond understanding the biological properties of PTH. It could indicate that oxidation of hormones changes the biological properties of these hormones. Many peptide hormones have methionines in their amino acid sequence. This could have similar consequences as it was shown for PTH. This applies also for FGF23 (amino acid structure of FGF23 see, Figure S3).

Using iPTH measurements to identify patients with secondary hyperparathyroidism as it was previously done in the EVOLVE trial might cause the selection of patients without true secondary hyperparathyroidism[42] and thus may contribute to a failure of such trials although the investigated drug is potentially effective. The data presented here, but also published clinical data on the subject,[13,14,28,43-46] make the use of iPTH assays for clinical purposes appear in a critical light. It may be currently more appropriate to measure bone alkaline phosphatase for clinical purposes until further studies allow establishment of guidelines for n-oxPTH. Oxidized forms of PTH are more likely a biomarker of oxidative stress. Oxidative stress is of course also harmful to CKD patients and will further promote disease progression. Treatment of oxidative stress, however, is for sure different from treating disturbances of PTH in CKD patients. Only assay systems that measure bioactive PTH are suitable to guide clinical decision making properly. The vast majority of PTH is oxidized and loses its bioactivity. PTH assays designed for clinical use should, however, measure bioactive PTH, because clinicians need this information to adjust properly medications such as calcimimetics, vitamin D, or phosphate binders. All these drugs are essential for CKD patients and interfere with the endocrine PTH system. Having a clinical laboratory tool that cannot really measure bioactive PTH makes clinical decision making rather difficult or even risky for patients.

ACKNOWLEDGMENT

Biomedica Medizinprodukte GmbH & Co KG provided the cFGF23 ELISA (BI-20702) and iFGF23 ELISA (BI-20700). We thank H. Froß for technical help. Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR CONTRIBUTIONS

B. Hocher designed research; B. Zhang, M. Feger, S. Rausch, U. Querfeld, T. Slowinski, and K. Horvathova performed research; C. Chu, Y. Xiong, and A.A. Hasan assisted with the data interpretation and manuscript preparation; and S. Zeng analyzed data and wrote the manuscript. F. Lang, M. Föller, B.K. Krämer, F. Schäfer, D. Haffner, and T.B. Dschietzig provided scientific advice and revised the manuscript.

REFERENCES

1. Kuro-o M, Moe OW. FGF23-alpha Klotho as a paradigm for a kidney-bone network. *Bone*. 2017;100:4-18.
2. Krajisnik T, Bjorklund P, Marsell R, et al. Fibroblast growth factor-23 regulates parathyroid hormone and 1alpha-hydroxylase

expression in cultured bovine parathyroid cells. *J Endocrinol*. 2007;195:125-131.
3. Isakova T., Cai X., Lee J., et al. Longitudinal FGF23 trajectories and mortality in patients with CKD. *J Am Soc Nephrol*. 2018;29:579-590.
4. Isakova T, Wahl P, Vargas GS, et al. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. *Kidney Int*. 2011;79:1370-1378.
5. Graciolli FG, Neves KR, Barreto F, et al. The complexity of chronic kidney disease-mineral and bone disorder across stages of chronic kidney disease. *Kidney Int*. 2017;91:1436-1446.
6. Lavi-Moshayoff V, Wasserman G, Meir T, Silver J, Naveh-Many T. PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. *Am J Physiol Renal Physiol*. 2010;299:F882-F889.
7. Masuyama R, Stockmans I, Torrekens S, et al. Vitamin D receptor in chondrocytes promotes osteoclastogenesis and regulates FGF23 production in osteoblasts. *J Clin Invest*. 2006;116:3150-3159.
8. David V, Martin A, Isakova T, et al. Inflammation and functional iron deficiency regulate fibroblast growth factor 23 production. *Kidney Int*. 2016;89:135-146.
9. Rodriguez-Ortiz ME, Lopez I, Munoz-Castaneda JR, et al. Calcium deficiency reduces circulating levels of FGF23. *J Am Soc Nephrol*. 2012;23:1190-1197.
10. Ferrari SL, Bonjour JP, Rizzoli R. Fibroblast growth factor-23 relationship to dietary phosphate and renal phosphate handling in healthy young men. *J Clin Endocr Metab*. 2005;90:1519-1524.
11. Feger M, Hase P, Zhang BB, et al. The production of fibroblast growth factor 23 is controlled by TGF ss 2. *Sci Rep*. 2017;7:4982.
12. Meir T, Durlacher K, Pan Z, et al. Parathyroid hormone activates the orphan nuclear receptor Nurr1 to induce FGF23 transcription. *Kidney Int*. 2014;86:1106-1115.
13. Hocher B, Oberthur D, Slowinski T, 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 Blood Press Res*. 2013;37:240-251.
14. Ursem SR, Vervloet MG, Hillebrand JJG, de Jongh RT, Heijboer AC. Oxidation of PTH: in vivo feature or effect of preanalytical conditions? *Clin Chem Lab Med*. 2018;56:249-255.
15. Hocher B, Armbruster FP, Stoeva S, et al. Measuring parathyroid hormone (PTH) in patients with oxidative stress—do we need a fourth generation parathyroid hormone assay? *PLoS One*. 2012;7:e40242.
16. Panah F, Ghorbanihaghjo A, Argani H, Zarmehri MA, Ahmad SNS. Ischemic acute kidney injury and klotho in renal transplantation. *Clin Biochem*. 2018;55:3-8.
17. Querfeld U, Anarat A, Bayazit AK, et al. The Cardiovascular Comorbidity in Children with Chronic Kidney Disease (4C) study: objectives, design, and methodology. *Clin J Am Soc Nephrol*. 2010;5:1642-1648.
18. Schwartz GJ, Munoz A, Schneider MF, et al. New equations to estimate GFR in children with CKD. *J Am Soc Nephrol*. 2009;20:629-637.
19. Bär L, Hase P, Föller M. PKC regulates the production of fibroblast growth factor 23 (FGF23). *PLOS ONE*. 2019:14(3):e0211309. http://dx.doi.org/10.1371/journal.pone.0211309.
20. Lowik CW, van der Pluijm G, Bloys H, et al. Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. *Biochem Biophys Res Commun*. 1989;162:1546-1552.
21. Greenfield EM, Gornik SA, Horowitz MC, Donahue HJ, Shaw SM. Regulation of cytokine expression in osteoblasts by parathyroid hormone: rapid stimulation of interleukin-6 and leukemia inhibitory factor mRNA. *J Bone Miner Res*. 1993;8:1163-1171.
22. Sakagami Y, Girasole G, Yu XP, Boswell HS, Manolagas SC. Stimulation of interleukin-6 production by either calcitonin gene-related peptide or parathyroid hormone in two phenotypically distinct bone marrow-derived murine stromal cell lines. *J Bone Miner Res*. 1993;8:811-816.
23. Keller H, Kneissel M. SOST is a target gene for PTH in bone. *Bone*. 2005;37:148-158.
24. Rhee Y, Allen MR, Condon K, et al. PTH receptor signaling in osteocytes governs periosteal bone formation and intracortical remodeling. *J Bone Miner Res*. 2011;26:1035-1046.
25. Kramer I, Loots GG, Studer A, Keller H, Kneissel M. Parathyroid hormone (PTH)-induced bone gain is blunted in SOST overexpressing and deficient mice. *J Bone Miner Res*. 2010;25:178-189.
26. Yang H, Dong J, Xiong W, Fang Z, Guan H, Li F. N-cadherin restrains PTH repressive effects on sclerostin/SOST by regulating LRP6-PTH1R interaction. *Ann N Y Acad Sci*. 2016;1385:41-52.
27. Doyon A, Fischer D-C, Bayazit AK, et al. Markers of bone metabolism are affected by renal function and growth hormone therapy in children with chronic kidney disease. *PLoS One*. 2015;10:e0113482.
28. Tepel M, Armbruster FP, Gron HJ, et al. Nonoxidized, biologically active parathyroid hormone determines mortality in hemodialysis patients. *J Clin Endocrinol Metab*. 2013;98:4744-4751.
29. Cheloha RW, Gellman SH, Vilardaga JP, Gardella TJ. PTH receptor-1 signalling-mechanistic insights and therapeutic prospects. *Nat Rev Endocrinol*. 2015;11:712-724.
30. 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. *J Biol Chem*. 1990;265:5671-5676.
31. Frelinger AL 3rd, Zull JE. The role of the methionine residues in the structure and function of parathyroid hormone. *Arch Biochem Biophys*. 1986;244:641-649.
32. Lopez I, Rodriguez-Ortiz ME, Almaden Y, et al. Direct and indirect effects of parathyroid hormone on circulating levels of fibroblast growth factor 23 in vivo. *Kidney Int*. 2011;80:475-482.
33. Liu SG, Tang W, Zhou JP, et al. Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. *J Am Soc Nephrol*. 2006;17:1305-1315.
34. Saji F, Shiizaki K, Shimada S, et al. Regulation of fibroblast growth factor 23 production in bone in uremic rats. *Nephron Physiol*. 2009;111:59-66.
35. Samadfam R, Richard C, Nguyen-Yamamoto L, Bolivar I, Goltzman D. Bone formation regulates circulating concentrations of fibroblast growth factor 23. *Endocrinology*. 2009;150:4835-4845.
36. Burnett-Bowie SAM, Henao MP, Dere ME, Lee H, Leder BZ. Effects of hPTH(1–34) infusion on circulating serum phosphate, 1,25-dihydroxyvitamin D, and FGF23 levels in healthy men. *J Bone Miner Res*. 2009;24:1681-1685.
37. Gutierrez OM, Smith KT, Barchi-Chung A, Patel NM, Isakova T, Wolf M. (1–34) parathyroid hormone infusion acutely lowers

fibroblast growth factor 23 concentrations in adult volunteers. *Clin J Am Soc Nephro*. 2012;7:139-145.
38. Wolf M. Forging forward with 10 burning questions on FGF23 in kidney disease. *J Am Soc Nephrol*. 2010;21:1427-1435.
39. Xu G, Luo K, Liu H, Huang T, Fang X, Tu W. The progress of inflammation and oxidative stress in patients with chronic kidney disease. *Ren Fail*. 2015;37:45-49.
40. Lang F, Leibrock C, Pandyra AA, Stournaras C, Wagner CA, Foller M. Phosphate homeostasis, inflammation and the regulation of FGF-23. *Kidney Blood Press Res*. 2018;43:1742-1748.
41. Ursem SR, Francic V, Keppel M, et al. The effect of vitamin D supplementation on plasma non-oxidised PTH in a randomised clinical trial. *Endocr Connect*. 2019;8(5):518-527.
42. Hocher B, Zeng SF. Need for better PTH assays for clinical research and patient treatment. *Clin Chem Lab Med*. 2018;56:183-185.
43. Lu YP, Zeng S, Chu C, et al. Non-oxidized PTH (n-oxPTH) is associated with graft loss in kidney transplant recipients. *Clin Chim Acta*. 2020;508:92-97.
44. Cavalier E, Lukas P, Warling X, Moonen M, Smelten N, Delanaye P. The percentage of non-oxidized PTH concentration remains stable over a period of 1 year in hemodialyzed patients. *Clin Chim Acta*. 2020;506:107-109.
45. Ursem SR, Vervloet MG, de Jongh RT, Heijboer AC. Oxidation of parathyroid hormone. *Clin Chim Acta*. 2020;506:84-91.
46. Ursem S, Francic V, Keppel M, et al. The effect of vitamin D supplementation on plasma non-oxidised PTH in a randomised clinical trial. *Endocr Connect*. 2019;8:518-527.
47. van der Velde M, Matsushita K, Coresh J, et al. Lower estimated glomerular filtration rate and higher albuminuria are associated with all-cause and cardiovascular mortality. A collaborative meta-analysis of high-risk population cohorts. *Kidney Int*. 2011;79:1341-1352.
48. Hayek SS, Sever S, Ko YA, et al. Soluble urokinase receptor and chronic kidney disease. *New Engl J Med*. 2015;373:1916-1925.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zeng S, Querfeld U, Feger M, et al. Relationship between GFR, intact PTH, oxidized PTH, non-oxidized PTH as well as FGF23 in patients with CKD. *The FASEB Journal*. 2020;00: 1–13. https://doi.org/10.1096/fj.202000596R

11. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

12. Complete List of Publications

1. **Zeng S**, Querfeld U, Feger M, Haffner D, Hasan AA, Chu C, Slowinski T, Bernd Dschietzig T, Schäfer F, Xiong Y, Zhang B, Rausch S, Horvathova K, Lang F, Karl Krämer B, Föller M, Hocher B. Relationship between GFR, intact PTH, oxidized PTH, non-oxidized PTH as well as FGF23 in patients with CKD. FASEB J. 2020 Nov;34(11):15269-15281. doi: 10.1096/fj.202000596R (IF= 4.966)

2. **Zeng S**, Slowinski T, Pommer W, Hasan AA, Gaballa MMS, Lu Y, Krämer BK, Hocher B. Sclerostin is an independent risk factor for all-cause mortality in kidney transplant recipients. Clin Exp Nephrol. 2020 Dec;24(12):1177-1183. doi: 10.1007/s10157-020-01956-y (IF=1.77)

3. Hocher B, **Zeng S**. Need for better PTH assays for clinical research and patient treatment. Clin Chem Lab Med. 2018;56(2):183–185. doi:10.1515/cclm-2017-0617 (IF= 3.638)

4. Hocher B, **Zeng S**. Clear the Fog around Parathyroid Hormone Assays: What Do iPTH Assays Really Measure? Clin J Am Soc Nephrol. 2018;13(4):524–526. doi:10.2215/CJN.01730218 (IF=6.243)

5. Lu YP, **Zeng S**, Chu C, Hasan AA, Slowinski T, Yin LH, Krämer BK, Hocher B. Non-oxidized PTH (n-oxPTH) is associated with graft loss in kidney transplant recipients. Clin Chim Acta. 2020 Sep; 508:92-97. doi: 10.1016/j.cca.2020.05.022. (IF= 2.615)

6. Cai S, Li J, Zeng S, Hu L, Peng Y, Tang S, **Zeng S**, Chu C, Gong F, Lin G, Hocher B. Impact of vitamin D on human embryo implantation-a prospective cohort study in women undergoing fresh embryo transfer. Fertil Steril. 2020 Oct 7:S0015-0282(20)32206-8. doi: 10.1016/j.fertnstert.2020.09.005. (IF= 6.312)

7. Bär L, Feger M, Fajol A, Klotz LO, **Zeng S**, Lang F, Hocher B, Föller M. Insulin suppresses the production of fibroblast growth factor 23 (FGF23). Proc Natl Acad Sci U S A. 2018 May 29;115(22):5804-5809. doi: 10.1073/pnas.1800160115. (IF= 9.58)

8. Hasan AA, von Websky K, Reichetzeder C, Tsuprykov O, Gaballa MMS, Guo J, **Zeng S**, Delić D, Tammen H, Klein T, Kleuser B, Hocher B. Mechanisms of GLP-1 receptor-independent renoprotective effects of the dipeptidyl peptidase type 4 inhibitor linagliptin in GLP-1 receptor knockout mice with 5/6 nephrectomy. Kidney Int. 2019 Jun;95(6):1373-1388. doi: 10.1016/j.kint.2019.01.010. (IF= 8.945)

9. Chu C, Hasan AA, Gaballa MMS, **Zeng S**, Xiong Y, Elitok S, Krämer BK, Hocher B.

Endostatin Is an Independent Risk Factor of Graft Loss after Kidney Transplant. Am J Nephrol. 2020;51(5):373-380. doi: 10.1159/000507824. (IF= 3.411)

10. Lu YP, Hasan AA, **Zeng S**, Hocher B. Plasma ET-1 Concentrations Are Elevated in Pregnant Women with Hypertension -Meta-Analysis of Clinical Studies. Kidney Blood Press Res. 2017;42(4):654-663. doi: 10.1159/000482004. (IF= 3)

11. Zhang X, Dong Y, Sun G, Hasan AA, Tian M, **Zeng S**, Li J, Zeng S, Ma J, Li J, Hocher B. Paternal Programming of Liver Function and Lipid Profile Induced by a Paternal Pre-Conceptional Unhealthy Diet: Potential Association with Altered Gut Microbiome Composition. Kidney Blood Press Res. 2019;44(1):133-148. doi: 10.1159/000497487. (IF= 1.898)

12. Luo T, Chen X, **Zeng S**, Guan B, Hu B, Meng Y, Liu F, Wong T, Lu Y, Yun C, Hocher B, Yin L. Bioinformatic identification of key genes and analysis of prognostic values in clear cell renal cell carcinoma. Oncol Lett. 2018 Aug;16(2):1747-1757. doi: 10.3892/ol.2018.8842. (IF= 1.871)

13. Zhang P, Zhong S, Wang G, Zhang SY, Chu C, **Zeng S**, Yan Y, Cheng X, Bao Y, Hocher B, Yang X. N-Acetylcysteine Suppresses LPS-Induced Pathological Angiogenesis. Cell Physiol Biochem. 2018;49(6):2483-2495. doi: 10.1159/000493874.

14. Wang G, Li PZ, Zhang SY, Zhong S, Chu C, **Zeng S**, Yan Y, Cheng X, Chuai M, Hocher B, Yang X. Lipopolysaccharides (LPS) Induced Angiogenesis During Chicken Embryogenesis is Abolished by Combined ETA/ETB Receptor Blockade. Cell Physiol Biochem. 2018;48(5):2084-2090. doi: 10.1159/000492547.

15. Lu YP, Reichetzeder C, Prehn C, Yin LH, Yun C, **Zeng S**, Chu C, Adamski J, Hocher B. Cord Blood Lysophosphatidylcholine 16: 1 is Positively Associated with Birth Weight. Cell Physiol Biochem. 2018;45(2):614-624. doi: 10.1159/000487118.

16. Lu YP, Hasan AA, **Zeng S**, Hocher B. Plasma ET-1 Concentrations Are Elevated in Pregnant Women with Hypertension -Meta-Analysis of Clinical Studies. Kidney Blood Press Res. 2017;42(4):654-663. doi: 10.1159/000482004. (IF= 3)

17. Yang F, Lai X, Deng L, Liu X, Li J, **Zeng S**, Zhang C, Hocher CF, Hocher B. Association of endothelin-1 gene polymorphisms with the clinical phenotype in primary nephrotic syndrome of children. Life Sci. 2014 Nov 24;118(2):446-50. doi: 10.1016/j.lfs.2014.04.010. (IF=3.052)

13. Acknowledgement

I would like to express my deepest appreciation to my supervisor Prof. Berthold Hocher and Prof. Wolfgang Pommer. I believe they are the great epitome of enthusiasm, persistence and dedication to science. I sincerely appreciate their support for sincere encouragement, dedicated support, constructive advice and care for life through my wonderful and unforgettable MD journal in Germany.

Many thanks to all authors of the publication for their efforts and support in this project. I would like to express my deepest gratitude to Prof. Bernhard Karl Krämer for his conceptual suggestions and revision of the manuscript.

I am sincerely grateful for the help of my colleagues and friends in our team, Ahmed A. Hasan, Yongping Lu, Mohamed M.S. Gaballa, Yingquan Xiong, Chang Chu, Yaochen Cao and Xin Chen. Thank you all so much for your support and the exchange of idea for my project along the way of my MD journey in last three years. Many thanks to Ahmed A. Hasan, without whose collegial and benevolent collaboration I couldn't have achieved this work. I am very grateful for his great commitment to supporting my doctorate and for his constant helpfulness. I would like to thank Mohamed M.S. Gaballa for the laboratory technical assistance.

Last but not least, I would like to thank my family, including my parents, my big brother, big sister and especially my husband Yao Xue who offered great love, endless support and understanding during my MD adventure in Berlin.

Berlin, Germany
2020.11.07