Assessment of 24,25(OH)2D levels does not support FGF23-mediated catabolism of vitamin D metabolites

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Abstract

Progressive elevations of fibroblastic growth factor 23 [FGF23] in chronic kidney disease may reduce serum 25-hydroxyvitamin D [25(OH)] and 1,25-dihydroxyvitamin D [1,25(OH)2D] levels, via stimulation of 24-hydroxylase (Cyp24A1) mediated catabolism of these vitamin D metabolites. To test this possibility, we measured serum concentrations of 24,25-dihydroxyvitamin D [24,25(OH)2D], a product of Cyp24A1 hydroxylation of 25(OH)D, in the Col4α3 knockout mouse, a model of Alport syndrome-derived chronic kidney disease, and in patients with chronic kidney disease of variable severity. There was an inverse correlation between serum FGF23 and both 25(OH)D and 1,25(OH)2D in the mouse model but no significant relationship was observed in the cross-sectional patient cohort. The FGF23-dependent increase in Cyp24a1 mRNA expression in the mouse kidneys was consistent with the possibility that FGF23 induces vitamin D catabolism. There was, however, a reduction in serum 24,25(OH)2D levels, rather than the expected elevation, in both the mice and patients with chronic kidney disease. Low 25(OH)D and elevated FGF23 and parathyroid hormone levels were correlated with the reduced serum 24,25(OH)2D concentrations of these patients. Thus, we failed to find support for FGF23-mediated catabolism of vitamin D metabolites in chronic kidney disease assessed by 24,25(OH)2D levels.

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Introduction

Chronic kidney disease (CKD) is associated with progressive elevations of serum PTH and FGF23 and reductions in 25(OH)D and $1,25(OH)_2D$ concentrations. The pathogenesis of CKD has been viewed as arising from abnormalities of the PTH-Vitamin D axis, where reductions in $1,25(OH)_2D$ leads to secondary increases in PTH [1]. The discovery of the FGF23-vitamin D endocrine network, however, has challenged this conceptual framework, by implicating FGF23 as the initial hormonal adaptive response leading to phosphaturia, reduced circulating $1,25(OH)_2D$ levels and secondary hyperparathyroidism [2–7].

FGF23 is a ~32 kDa secreted protein, predominately expressed in osteoblasts and osteocytes in bone [8–11], that principally targets receptor complexes consisting of FGFR 1, 3 or 4 and the transmembrane β glucuoronidase, α-Kl [12–15] located in the kidney. PTH and FGF23 have concordant effects to promote phosphaturia by suppressing phosphate co-transporters Npt2a and Npt2c expression, but have opposite effects on vitamin D metabolism, where PTH stimulates Cyp27b1-mediated conversion of 25(OH)D to $1,25(OH)_2D$, and FGF23 inhibits Cyp27b1 activity and stimulates Cyp24a1, the enzyme responsible for conversion of 25(OH)D and $1.25(OH)_2D$ to inactive $24,25(OH)_2D$ and $1,24,25(OH)_3D$ metabolites, respectively [16–22]. $1,25(OH)_2D$ also stimulates FGF23 production in bone to create a bone-kidney endocrine feedback loop [23].

The primary role of FGF23 in the pathogenesis of abnormal vitamin D metabolism and pathogenesis of secondary hyperparathyroidism in CKD, however, remains unproven, in part because the contribution of FGF23-mediated suppression of Cyp27b1 and stimulation Cyp24a1 activity to the observed reductions in serum 25(OH)D and $1.25(OH)_2D$ levels in CKD has not been defined. In addition, while FGF23-mediated suppression of Cyp27b1-mediated synthesis of $1,25(OH)_2D$ is supported by concordant changes in FGF23, Cyp27b1 expression and reductions in serum $1,25(OH)_2D$ levels in hereditary models of excess FGF23, the competing effects of PTH and FGF23 on Cyp27b1 expression and activity in CKD creates a more complex regulatory environment. Measurement of Cyp27b1 and Cyp24A1 enzymatic activity in a diseased kidney is technically challenging, but assays are available to measure both $1,25(OH)_2D$ and $24,25(OH)_2D$, the end products of Cyp27b1 and Cyp24a1-mediated hydroxylation, respectively.

In the current study, to test the contributions of Cyp27b1 mediated suppression and Cyp24a1-mediated catabolism of vitamin D metabolites in CKD, we assessed serum 25(OH)D, $1,25(OH)_2D$ and $24,25(OH)_2D$ levels in both a mouse model of CKD [7] and in a cohort of humans with impaired renal function of varying severity. We predicted that circulating $24,25(OH)_2D$ concentrations should be increased and 25(OH) and $1,25(OH_2D$ decreased proportionate to the degree of renal insufficiency, if FGF23 stimulation of Cyp24a1 and inhibition of Cyp27b1 are contributing to the abnormalities of vitamin D metabolism in CKD.

Results

Serum Abnormalities in Col4a3–/– mice (Table 1)

Consistent with our prior characterization of the Col4a3^{-/-} model of CKD [7], we found that by 12-weeks of age, Col4a3^{-/-} mice had significant elevations of serum BUN and creatinine concentrations. Col4a3^{-/-} mice also had elevated serum phosphate levels and showed marked elevations of both circulating PTH and FGF23 concentrations compared to wild-type controls. The fractional excretion of phosphate was also increased ~3-fold in Col4a3^{-/-} mice. We found that serum BUN and creatinine positively correlated with serum FGF23 levels (Fig 1A and B). In addition, serum FGF23 positively correlated with serum PTH concentrations (Fig 1C). The fractional excretion of phosphate also correlated with serum FGF23 levels (Fig 1D). Serum calcium and fractional excretion of calcium were also increased in Col4a3^{-/-}, suggesting an advanced stage of hyperparathyroidism. Serum alkaline phosphatase was slightly increased in Col4a3^{-/-} mice, likely reflecting the high turnover bone disease that has been previously characterized in greater detail in this CKD mouse model [7].

Abnormalities in vitamin D metabolism in Col4a3^{-/-}

Next, we assessed serum vitamin D metabolite concentrations in Col4a3^{-/-} and wild-type mice (Table 2). We found that serum 25(OH)D levels were significantly reduced in Col4a3^{-/-} mice as were the serum $1,25(OH)_2D$ levels, concordant with our prior findings in these mice [7]. We have also found, that elevations in serum FGF23 were inversely correlated with both 25(OH)D and $1,25(OH)_2D$ serum concentrations in Col4a3^{-/-} mice (Fig 1E and F). In a multiple regression analysis, which included serum FGF23, PTH, $1,25(OH)_2D$, BUN, phosphate and calcium, only FGF23 predicted the changes in 25(OH)D (p=0.02, β= −1.54, std error, 0.46).

To explore mechanisms for the observed alterations in serum 25(OH)D, we measured the expression of Cyp27b1 in the kidney, the enzyme responsible for conversion of 25(OH)D to $1,25(OH)_2D$. The message levels of Cyp27b1 were not different between wild-type and Col4a3^{-/-} at 6 and 8 weeks of age, but were increased ~3-fold in Col4a3^{-/-} mice compared to controls (Fig 2A) by 10 wk-old animals and further increased with renal damage progression. Thus, Cyp27b1 mRNA may not reflect enzyme activity as previously reported [24]. Thus, we found no evidence for a decrease in the expression of transcripts encoding the enzymes responsible for producing 25(OH) or $1,25(OH)_2D$.

To further investigate the possibility of increased catabolism as an explanation for reduction in circulating vitamin D metabolites, we assessed the expression of Cyp24a1 message levels, the enzyme that converts 25(OH)D and $1,25(OH)_2D$ into 24-hydroxylated derivatives that are converted to calcitroic acid. Similar to Cyp27b1, Cyp24a1 expression was not different between wild-type and Col4a3[v]mice at 6 and 8 weeks of age, but was increased in the kidneys of Col4a3^{-/-} mice compared to wild-type controls at 10 and 12 weeks-of-age (Fig 2B). The relative increase in Cyp24a1 (~10 fold) was greater than Cyp27b1 (~3 fold) resulting in a higher ratio of Cyp24a1/Cyp27b1 in Col4a3^{-/-} mice by 10 weeks-of-age. To determine the contribution of FGF23 to the alterations in Cyp27b1 and Cyp24a1 message,

we examined expression of Cyp27b1 and Cyp24a1 message in the kidney after genetic ablation of FGF23 in Col4a3$^{-/-}$ mice. We found that compared to Col4a3$^{-/-}$ mice, that loss of FGF23 in compound Fgf23$^{-/-}$ Col4a3$^{-/-}$ mice dramatically increased Cyp27b1 expression, indicating that FGF23, was responsible for maintaining Cyp27b1 at a relative low-level (Fig 2C). More importantly, in the same setting, ablation of FGF23 restored Cyp24a1 mRNA levels (Fig 2D), proving evidence that FGF23 is likely responsible for up-regulation of this enzyme.

To seek additional evidence for enhanced vitamin D catabolism, we measured circulating 24,25(OH)$_2$D levels in Col4a3$^{-/-}$ and wild-type mice as an index of Cyp24a1 activity. Surprisingly, we found that 24,25(OH)$_2$D levels were significantly lower in Col4a3$^{-/-}$ mice than wild-type controls (Table 2). In addition, we found a strong negative correlation between serum FGF23 and serum 24,25(OH)$_2$D levels in pooled data from Col4a3$^{-/-}$ and wild-type mice (Fig 2E). These results are opposite to those anticipated if serum 24,25(OH)$_2$D concentrations are an indicator of FGF23-mediated Cyp24a1 hydroxylation of 25(OH)D.

Finally, we measured in Col3a4$^{-/-}$ and wild-type mice the message expression in the liver of two enzymes responsible for 25(OH)D production, Cyp2r1 and Cyp27a1. We found that both transcripts were slightly, but not significantly, increased in Col3a4$^{-/-}$ mice compared to wild-type controls (Fig 2F).

Relationships between FGF23, PTH, and vitamin D metabolism in humans

We stratified age- and disease matched male patients with low 25(OH)D levels into two groups based on an eGFR threshold of < 60 ml/min/1.73 m^2 (Table 3). The eGFR, urine albumin creatinine ratio, serum FGF23, serum PTH and fractional excretion of phosphate values were significantly greater in the group with eGFR<60 ml/min/1.73 m^2 compared to the patients with eGFR>60 ml/min/1.73 m^2. As expected based on the selection criteria, we observed no difference in serum 25(OH)D levels in the two groups, both of which had average values below the lower limit of normal. Serum 1,25(OH)$_2$D concentrations, however, were in the normal range and did not differ between the two groups.

We found a strong negative correlation between eGFR and FGF23 (Fig 3A) and a positive correlation between serum creatinine and FGF23 (Fig 3B), as well as a strong positive correlation between serum FGF23 and PTH levels (Fig 3C). We also observed a positive correlation between FGF23 and fractional excretion of phosphate (Fig 3D). Unlike the mouse model of CKD, however, we did not find a significant correlation between FGF23 and either 25(OH)D (Fig 3E) or 1,25(OH)$_2$D levels (Fig 3F) in the cohort of humans subjects, possibly due to the low circulating 25(OH)D levels used to select these patients. When we examined patients with FGF-23 levels >100 ng/ml (i.e., level typical of more advanced CKD patients), FGF23 did negatively correlate with baseline 1,25(OH)$_2$D levels (r= 0.56, P = 0.02, n = 14).

Similar to the findings in the mouse CKD model, however, serum 24,25(OH)$_2$D levels were significantly lower in patients with eGFR<60 ml/min/1.73 m^2 compared to patients with

eGFR 60 ml/min/1.73 m^2. Indeed the serum 24,25 (OH)$_2$D levels were 2-fold lower in the CKD group compared to the group with "normal" renal function (i.e., 0.65 vs 1.24 ng/ml).

Overall, the serum 24,25(OH)$_2$D levels, 1,25(OH)$_2$D, and FGF23 were 15-, 2-, and 1.6- fold higher, and PTH ~50% lower, respectively, in wild-type mice compared to humans with eGFR 60 ml/min/1.73 m^2. In the setting of impaired renal function, these same parameters were 8-, 1.6-, 8- and 11-fold higher, respectively, in the Col4a3$^{-/-}$ mice with CKD compared to subjects with eGFR<60 ml/min/1.73 m^2. Compared to controls, the reduction in the 24,25 (OH)$_2$D levels was 4-fold greater and the elevation in FGF23 and PTH were ~4-fold higher observed the mouse model of CKD than the respective changes in humans with CKD, suggesting that the mouse model is more sensitive alterations in mineral metabolism markers. This finding is similar to the accentuated alterations in FGF23 and vitamin D metabolism observed in Hyp mice compared to human patients with X-linked Hypophosphatemic Rickets [25].

To determine predictors of serum 24,25(OH)$_2$D levels in the human subjects, we performed univariate (Table 4, Fig 4) and multivariable regression analysis (Table 5). Serum PTH, 25(OH)D, FGF-23, creatinine and urinary fractional excretion of phosphorous were significant predictors of serum 24,25(OH)$_2$D levels in simple regression analysis (Table 4). An inverse relationship was present between FGF23, PTH and creatinine and 24,25(OH)D$_2$ levels (Fig 4). In contrast, a positive association was observed between serum 25(OH)D and 24,25(OH)$_2$D levels, suggesting that low substrate leads to low 24,25(OH)$_2$D levels. These predictor variables along with the interaction terms between them were utilized in multiple regression model. Collinearity statistics for all of the predictor variables entered in the multiple linear regression analysis indicated that in all cases, tolerance values were >0.7 and the VIF were <2. This excludes any serious problem with multicollinearity.

The final model predicting serum 24,25(OH)$_2$D level consisted of FGF-23, PTH, and 25(OH)D without any interaction terms (Table 5). These variables together explained 55% of the variations in serum 24,25(OH)$_2$D level (R^2_{adj} = 0.5548), and the overall relationship was significant $F_{(3, 24)}$ = 10.97, P = 0.0002. With other variables held constant, serum 24,25(OH)$_2$D level is negatively related to FGF-23 (t_{21} = −2.26, P = 0.0349) and PTH (t_{21} = −2.55, P = 0.0185), and positively related to 25(OH)D (t_{21} = 3.40, P = 0.0027). The final model hold the following equation: estimated 24,25(OH)$_2$D = −0.14307 + 0.03157 × 25(OH)D − 0.00126 × PTH − 0.00236 × FGF-23. The model suggested that a rise in serum PTH and FGF23 by 10 pg/mL would decrease 24,25(OH)$_2$D levels by 1% and 2% respectively. On the other hand a 10 ng/mL rise in serum 25(OH)D level would increase 24,25(OH)$_2$D level by 32%.

Discussion

In the current studies, we measured serum concentrations of 24,25(OH)$_2$D levels and assessed the relationships between serum 24,25(OH)$_2$D and FGF23, PTH, 25(OH)D, and 1,25(OH)$_2$D concentrations in both mice and humans with CKD to determine if FGF23 stimulates the catabolism of 25(OH)D and 1,25(OH)$_2$D in CKD[2, 26, 27]. Our initial findings supported a potential role of FGF23 in the reductions of 1,25(OH)$_2$D as well as 25(OH)D in

CKD. First, the ratio of Cyp24a1 to Cyp27b1 message is increased in the kidney of Colf4a3$^{-/-}$ mice. Second, serum FGF23 levels were inversely correlated with both serum 25(OH)D and 1,25(OH)$_2$D in Col4a3$^{-/-}$ mice, consistent with FGF23-meidated degradation of these metabolites. Interestingly, we did observe an inverse correlation between serum FGF23 and 1,25(OH)D, but not 25(OH)D levels, in the human subjects examined in this study. However, others have observed a correlation between decreased eGFR and reduced 25(OH)D and increased FGF23 concentrations [28, 29]. We were unable to find direct evidence, however, for increased Cyp24a1 activity in Col4a3$^{-/-}$ mice or the human subjects with CKD. Indeed, serum 24,25(OH)$_2$D levels, the initial product of Cyp24a1 mediated hydroxylation of 25(OH)D, were suppressed in Col4a3$^{-/-}$ mice and human subjects with CKD, rather than elevated.

The most conservative interpretation of these findings is that reductions in serum 25(OH)D and 1,25(OH)$_2$D in CKD are not caused by FGF23-mediated stimulation of Cyp24a1 catabolism. In this scenario, the increase in expression of Cyp24a1 message level in the kidney does not represent an increase in enzymatic activity. One possible explanation may be that elevated PTH decreases Cyp24a1 mRNA stability [30]. Consistent with this possibility, the message expression of Cyp27b1 also does not correlate with enzymatic activity in other states of combined PTH and FGF23 excess[24]. If so, low circulating levels 1,25(OH)$_2$D and 24,25(OH)$_2$D in CKD might result from a decreased total enzymatic activity in the diseased kidney, which is the principal source of circulating 24,25(OH)$_2$D [31]. Alternatively, a more speculative conclusion is that Cyp24a1 activity is increased in CKD in response to increased serum FGF23, but that serum 24,25(OH)$_2$D is not elevated due to its rapid clearance and conversion to calcitroic acid [32]. If so, 24,25(OH)$_2$D may not be a marker of increased Cyp24a1 activity in CKD. Consistent with this possibility, chronic 1,25(OH)$_2$D administration to rats *in vivo* increases the *in vitro* conversion of 25(OH)D to 24,25(OH)$_2$D, but at the same time decreases circulating 24,25(OH)$_2$D levels [33]. Further studies will be needed to distinguish between these two interpretations.

Limited 25(OH)D substrate might also influence the production of 24,25(OH)$_2$D [34]. In multiple regression analysis in human subjects, low 25(OH)D was a strong predictor of reductions in 24,25(OH)$_2$D. We found no alterations in hepatic enzymes that generate 25(OH)D. Interestingly, serum 24,25(OH)$_2$D levels were markedly higher in wild-type mice than humans with normal renal function. This may be due to genetic differences between mice and humans. Alternatively, the relatively high dietary content of vitamin D in rodent food may account for the higher 24,25(OH)$_2$D levels in both CKD and wild-type mice compared to humans with CKD and preserved renal function. Finally, reductions in serum 25(OH)D as a function of CKD severity might also result from the failure of a diseased kidney to recycle 25(OH)D from the glomerular ultrafiltrate back into proximal tubular cells for 1,25(OH)$_2$D synthesis, and back into the circulation to maintain serum 25(OH)D levels.

The finding of low levels of 24,25(OH)$_2$D in CKD has also been found in patients with CKD by Ishimura et al [34] and in the Seattle Kidney Study cohort (Ian H. DeBoer, personal communication). The clinical significance of low serum 24,25(OH)$_2$D concentrations is not clear. 24,25(OH)$_2$D may have a role in bone mineralization and fracture repair [35]. The low 24,25(OH)$_2$D was associated with increased mortality in the Seattle Kidney Study cohort,

but this could also reflect the effects of FGF23, which was a determinant of $24,25(OH)_2D$ levels and is associated with increased mortality in CKD [36].

Our results suggest a complex interplay between elevated PTH and FGF23 on Cyp27b1 functions in CKD. In this regard, we found that the kidney expression of Cyp27b1 was 4-fold increase in the $Col4a3^{-/-}$ CKD model, possibly reflecting actions of elevated PTH to stimulate Cyp27b1 expression, but Cyp27b1 was increased over 50-fold after ablation of FGF23 in Col4a3−/− mice. In spite of increased PTH, a negative correlation was observed between FGF23 and $1,25(OH)_2D$ levels in $Col4a3^{-/-}$ mice, consistent with the possibility that FGF23 and PTH have opposite effects on the production of $1,25(OH)_2D$. Other reports indicate that the stimulatory effects of PTH may be offset by the suppressive effects of FGF23 on Cyp27b1 enzymatic activity at the posttranscriptional levels [7, 24, 26]. The importance of FGF23 on vitamin D metabolism is supported by the observation that treatment of a rat model of anti-GBM nephritis with a neutralizing anti-FGF23 antibody increases serum $1,25(OH)_2D$ levels and Cyp27b1 expression and reduces Cyp24a1 expression [27].

Our findings suggest that FGF23 and PTH have concordant effects on phosphate homeostasis, since FGF23 is positively correlated with parathyroid hormone in both a homogenous mouse model of CKD and a more diverse group of human subjects with varying degrees of renal impairment. This positive association between PTH and FGF23 is consistent with other reports showing a strong association between elevated FGF23 levels and the severity of HPT in CKD and other disorders [13, 37, 38]. The mechanism of FGF23 and PTH co-regulation are unclear, and both direct effects of PTH to stimulate FGF23 expression in bone, indirect effects of PTH to stimulate FGF23 through PTH-mediated increase in $1,25(OH)_2D$ levels, and FGF23 effects to suppress PTH directly or to indirectly stimulate PTH due to FGF23-mediated suppression of $1,25(OH)_2D$ have been proposed [39].

Our study has several limitations. With regard to the mouse studies, we lack measurements of Cyp27b1 or Cyp24a1 enzymatic activity and important genetic and dietary differences in these models may limit extrapolating findings to humans. Observations in the mouse CKD model also highlight the conflicting regulatory networks, where initial FGF23-mediated suppression of $1,25(OH)_2D$ may lead to progressive elevations in PTH, which in turn may further stimulate both FGF23 and $1,25(OH)_2D$ levels. Further studies are needed to assess the interdependent regulation of PTH, FGF23 and $1,25(OH)_2D$ in CKD. With regards to the human studies, the sample size is small, the patient population is heterogeneous, and were preselected based on low 25(OH)D levels that may have lead to a failure to observe relationships between FGF23, PTH and vitamin D metabolites that are functionally important. However, the number of patients examined is insufficient for the accurate assessment of all the potential predictors of $24,25(OH)_2D$ levels. Our data in both mice and human models of CKD fail to explain the reductions in 25(OH)D levels, which was measured by immunoassay, which may also detect other vitamin D metabolites[40]. Perhaps assessment of 25(OH)D by HPLC or free 25(OH)D might provide different results. We need additional assessments of pathways leading to 25(OH)D production as well as vitamin D catabolic pathways, such as $1,24,25(OH)_3D$ levels, $24,25(OH)_2D$ levels after treatment with

vitamin D analogues and calcitroic acid (1α-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D) production[41].

In summary, we found that the measurements of $24,25(OH)_2D$ levels fail to evaluate the contribution of FGF23-driven increases in 24-hydroxylase in 25(OH)D catabolism in CKD. Indeed, circulating $24,25(OH)_2D$ levels were not elevated in either mice or humans with CKD. The significance of this unexpected negative finding remains to be established.

Methods

Animals and genotyping

All mice were maintained on a standard diet (7912, Harlan Teklad, Madison, WI, USA). Animal care and protocols were in accordance with the guidelines established by the University of Tennessee Institutional Animal Care and Use Committee as detailed in the "Guide for Care and Use of Laboratory Animals," prepared by the Institute on Laboratory Animal Resources, National Research Council (Department of Health & Human Services Publication NIH 86-23, National Academy Press, 1996).

Heterozygous Col4a3^{+/−} mice were obtained from Jackson Laboratories (Westgrove, PA, USA). Ear biopsies were collected to genotype the mice. REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) was used for DNA extraction and PCR amplification. Mice were genotyped for col4a3 mutation and PCR was repeated in all mice after sacrifice to exclude artifacts and ensure the correct genotype [9].

Serum biochemistry

Serum samples from mice were collected by intracardiac exsanguination. Calcium was measured using a Calcium CPC Liquicolor Kit (Stanbio Laboratories, Boerne, TX, USA) and phosphorus was measured using the phosphomolybdylate-ascorbic acid method [9]. Parathyroid hormone (PTH) levels were measured using the Mouse Intact PTH ELISA kit (Immutopics, Carlsbad, CA, USA). $1,25(OH)_2D$ and 25OHD levels were measured using the vitamin D EIA Kits (Immunodiagnostic Systems, Fountain Hills, AZ, USA).

FGF23 levels were measured in both mice and humans using the FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan). $24,25(OH)_2D$ was measured in both mice and human samples by Heartland Assays LLC (Ames, Iowa, USA) according to previously published procedures [31, 42]. 25(OH)D levels were measured in humans by immunochemical illuminometric assay, albumin by bromcresol green assay and creatinine by isotope dilution mass spectrometry traceable Jaffé method from Roche (compensated Jaffé, Roche Diagnostics, Mannheim, Germany).

RT-PCR

Total RNAs were isolated using TRI-reagent and first-strand cDNA was synthesized from the kidney or liver RNAs according to previously published method [43]. The iCycler iQ Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) were used for real-time quantitative PCR analysis. The expression was normalized by

glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) in the same sample and expressed as fold change of WT.

Human Subjects

The human cohort in the trial includes 53 patients with vitamin D deficiency [serum 25(OH)D level < 20 ng/ml] identified between April 2009 to July 2010. The study was approved by the institutional review board of Veterans Affairs Medical Center, Memphis, TN. Inclusion criteria for the study were the following: Men and women over the age of 18 years and serum 25(OH)D levels < 20 ng/ml. Exclusion criteria included: eGFR < 15 ml/min/1.73 m^2 or chronic dialysis patients, receipt of any form of vitamin D supplementation, presence of cirrhosis, sarcoidosis, lymphoma, malabsorption syndrome, solid organ transplant, or hypoparathyroidism, use of medications known to alter vitamin D metabolism, including rifampin, corticosteroids, anti-epileptics, phosphate binders, and calcimimetics. Patients with CKD were matched with patients with normal renal function based on several matching variables in 1:1 basis. The matching variables were age (within 5 years), race, gender, and diabetes mellitus (DM). Fourteen patients with CKD were matched with 14 patients with normal renal function.

Urinary protein excretion was measured as a ratio of spot urine protein-creatinine (UPC) ratio or as spot urine albumin-creatinine ratio (ACR) [ACR = $UPC^{(1.054)} \times 0.596$]. The eGFR was calculated according to the 4-variable formula used in the Modification of Diet in Renal Disease Study. Body mass index (BMI) was calculated by person's weight in kilograms divided by their height in square meters.

Statistical Analysis

For the mouse studies differences among the two groups were tested by Student T test using the Statistica software (Statsoft, Tulsa, OK, USA). The differences were considered statistically significant at p<0.05. Pearson correlations and multiple regression analysis were performed to examine the relationship between the measured serum parameters.

For the human subjects, continuous variables were presented as means and standard deviations (SD) and categorical variables as percentages, unless otherwise specified. Normally distributed continuous variables were compared using unpaired t-test and non-normally distributed continuous variables with Mann-Whitney U test. To evaluate the factors affecting the 24,25 (OH)2D level, univariate analyses were done using the following predictor variables: age, race, diabetes mellitus (DM), BMI, serum calcium, phosphorous, creatinine, FGF-23, PTH, 25(OH)D, 1,25(OH)2D, eGFR, and urine ACR level. The variable, 24,25(OH)2D was log transformed to attain normal distribution. All tests were 2-sided and P value < 0.05 was considered significant. Statistical analysis was conducted using SAS® version 9.2 (SAS Institute Inc., Cary, NC, U.S.A.).

Acknowledgments

Abbreviations

24,25(OH)$_2$D	24,25-dihydroxyvitamin D
1,25(OH)$_2$D	1,25-dihydroxyvitamin D
calcitriol	1α,25-Dihydroxycholecalciferol
25(OH)D	25-hydroxyvitamin D
calcidiol	25-hydroxychoelcalciferol
cholecalciferol	vitamin D
Cyp27b1	1α-hydroxylase
Cyp24a1	24-hydroxylase

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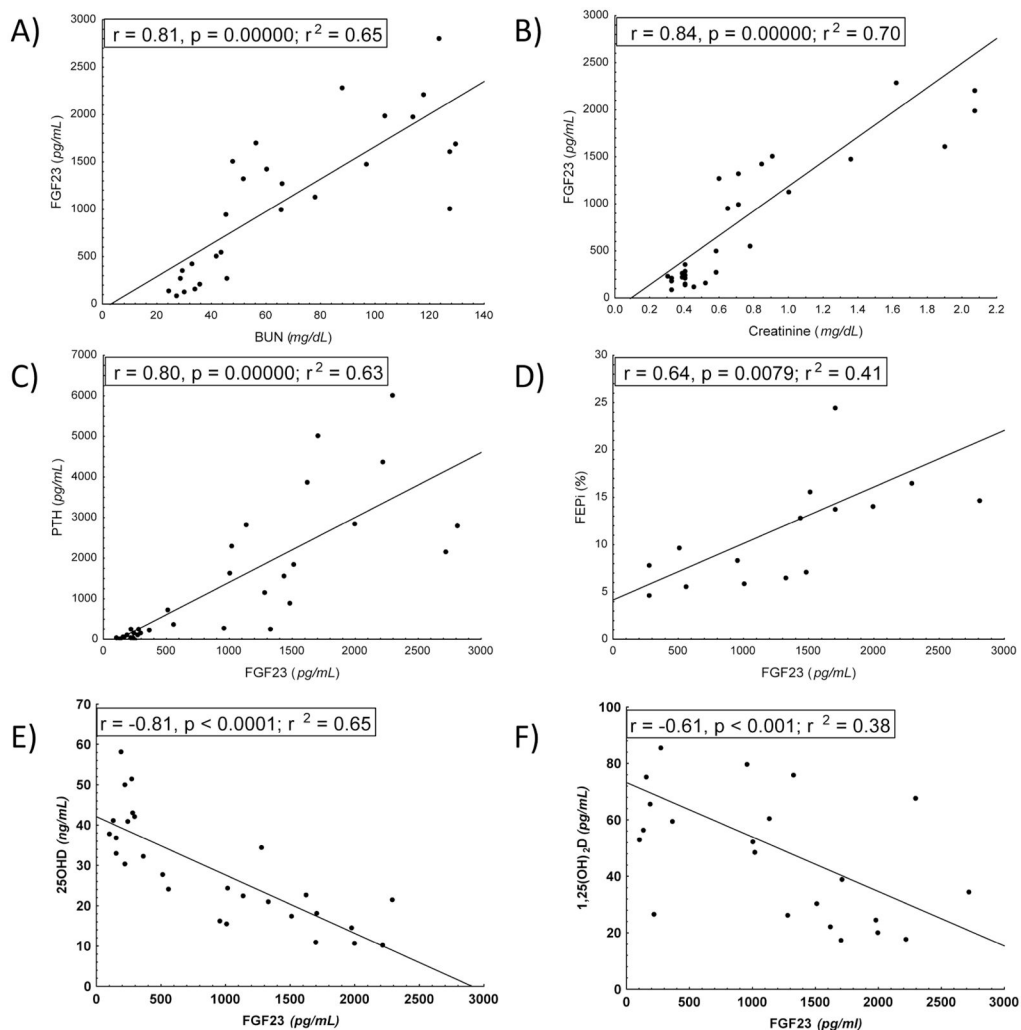**Figure 1. Pearson correlations between serum biochemical parameters in 12-week-old wild-type and Col4a3$^{-/-}$ mice**

Serum FGF23 was positively correlated with increments in serum BUN (A) and creatinine (B). Serum PTH and FGF23 were positively correlated (C). FGF23 was positively correlated with the fractional excretion of phosphate (D). Serum FGF23 levels were inversely correlated with serum 25(OH)D (E) and 1,25(OH)$_2$D (E).
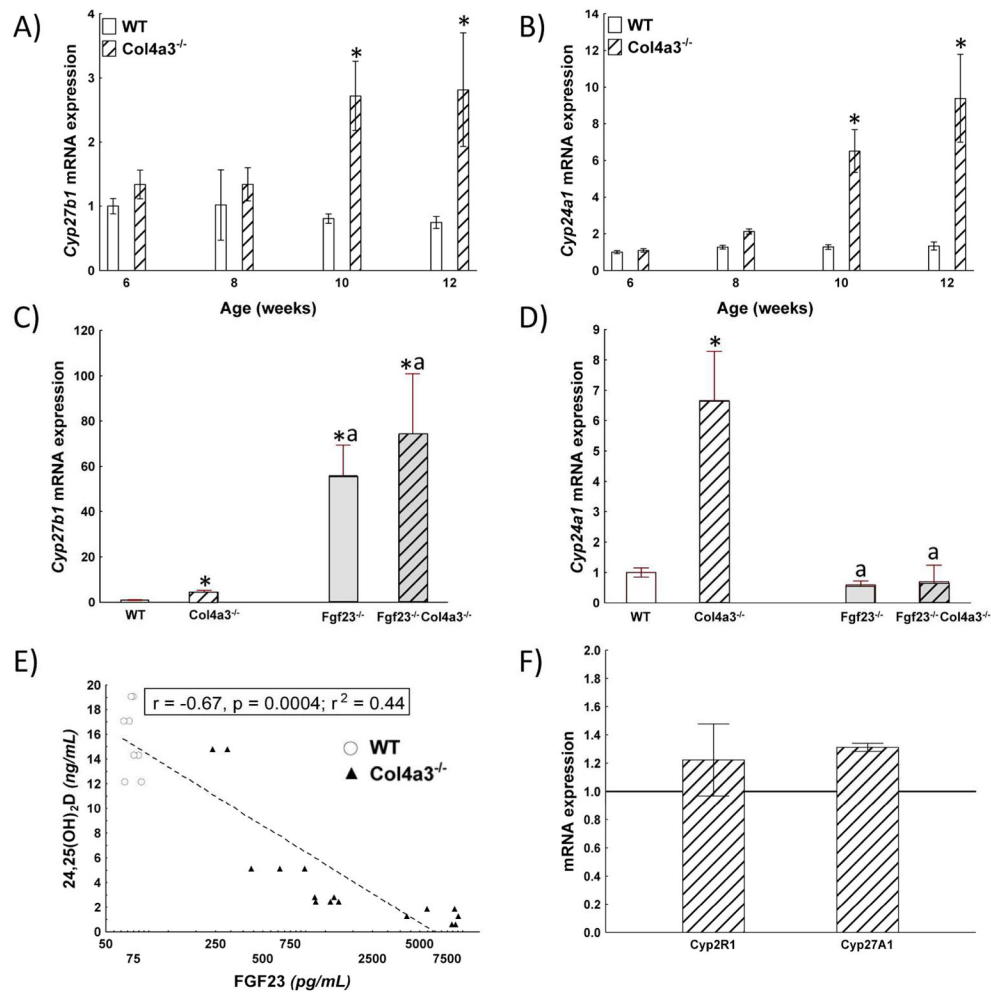
Figure 2. Regulation of vitamin D metabolism in 12-week-old wild-type and Col4a3$^{-/-}$ mice
A,B) Time-course expression of A) Cyp27b1 and B) Cyp24A in the kidney of WT and
Col4a3$^{-/-}$ mice. Both Cyp27b1 and Cyp24a1 message expression were age-dependently
increased in Col4a3$^{-/-}$ mice compared to wild-type controls. C, D) Expression of C)
Cyp27b1 and D) Cyp24A in the kidney of 12 week-old WT, Col4a3$^{-/-}$ Fgf23$^{-/-}$ and
compound Fgf23$^{-/-}$ ol4a3$^{-/-}$ mice. N 4, p<0.05, * vs. WT, a vs. Col4a3$^{-/-}$. mRNA
expression normalized by GAPDH and relative to values in wild-type controls. E)
Relationship between serum FGF23 and 24,25(OH)$_2$D levels. There was a significant
inverse correlation between serum FGF23 and serum 24,25(OH)$_2$D levels. (circles, wild-
type, triangles, Col4a3$^{-/-}$ mice) F) Expression of Cyp2R1 and cyp27A1 in the liver. mRNA
expression normalized by GAPDH and relative to values in wild-type controls. There was
no significant difference in expression of the enzymes that generate 25(OH)D in Col4a3$^{-/-}$
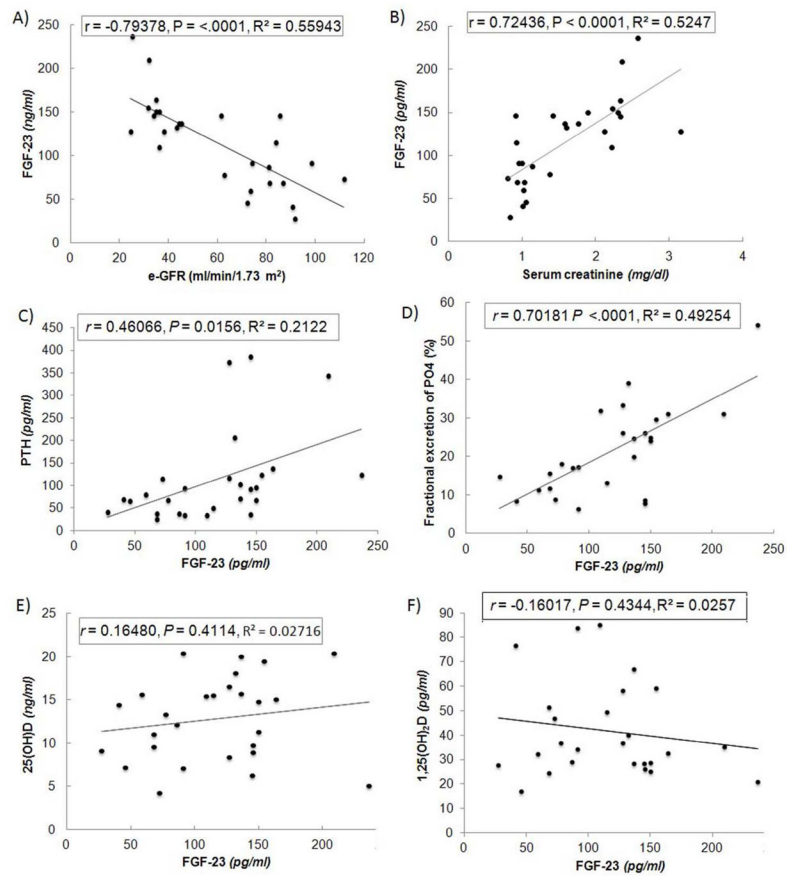mice compared to wild-type mice.

Figure 3. Regression analysis in human subjects with varying degrees of renal impairment
FGF23 is inversely correlated with eGFR (A). and positively correlated with serum creatinine (B). Serum PTH and FGF23 are positively correlated (C). FGF23 is also positively correlated with the fractional excretion of phosphate (D). Neither 25(OH)D (E) or 1,25(OH)$_2$D (F) are significantly correlated with FGF23.
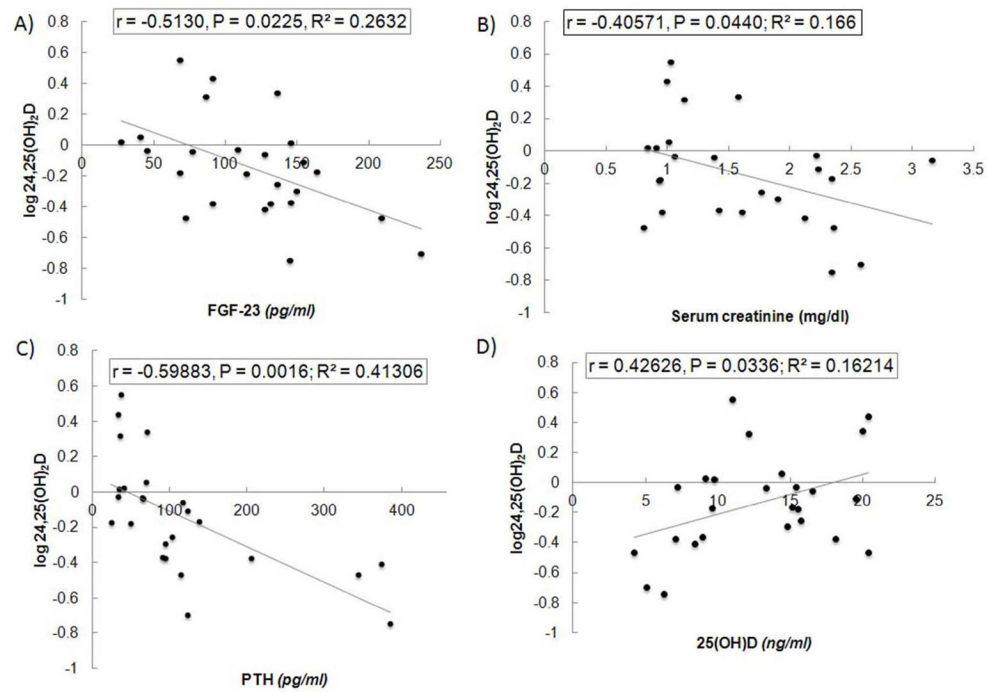
Fig 4. Determinants of serum 24,25(OH)₂D levels in human subjects

Serum 24,25(OH)2D was positively correlated with 25(OH)D (A) and inversely related to serum PTH (B), FGF23 (C), serum creatinine (D) and fractional excretion of phosphate (D).

Table 1

Serum biochemistries of in mice.

	WT	**Col4a3$^{-/-}$**
BUN (mg/dL)	19.9 ± 0.7	59.3 ± 9.2*
Serum creatinine (mg/dL)	0.45 ± 0.03	0.83±0.16*
Serum PTH (pg/mL)	34 ± 5	1827± 479*
Serum FGF23 (pg/mL)	135± 10	1216± 199*
Serum PO4 (mg/dL)	6.5 ± 0.5	9.5 ± 0.5*
Serum Ca (mg/dL)	8.8 ± 0.4	9.3 ± 0.3
Fractional excretion of PO$_4$(%)	4.7 ±1.6	14.8 ±2.7*
Fractional excretion of Ca (%)	1.4 ± 0.3	13.7 ± 4.0
Alkaline phosphatase (IU/L)	67.0 ± 8.6	90.2 ± 10.9

Values are expressed as mean ± SEM from at least 13 mice per group. Comparisons were performed using one-way ANOVA and post-hoc Fisher test. P<0.05 vs: (*) WT. BUN, blood urea nitrogen; PTH, parathyroid hormone; FGF-23, fibroblast growth factor-23; PO4, phosphorous; Ca, calcium.

Table 2

Serum vitamin D metabolite concentrations in mice.

	WT	Col4a3$^{-/-}$
25(OH)D (ng/ml)	44.1+3.2	24.8 ± 2.6*
1,25(OH)$_2$D (pg/ml)	101± 19	70 ± 19*
24,25(OH)$_2$D (ng/mL)	15.7 ± 1.0	4.1 ± 1.1*

Values are expressed as mean ± SEM from at least 13 mice per group. Comparisons were performed using one-way ANOVA and post-hoc Fisher test. $P < 0.05$ vs: (*) WT. 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)2D, 1,25-dihydroxyvitamin D; 24,25(OH)2D, 24,25-dihydroxyvitamin D.

Table 3

Serum biochemical parameters in patients grouped by eGFR.

	eGFR ≥ 60 (ml/min/1.73 m²) (*n = 14*)	eGFR < 60 (ml/min/1.73 m²) (*n = 14*)	*P* value
African American (%)	55	44	0.6946
Diabetes mellitus (%)	42	42	1
Age (years)	62.57 ± 6.46	65.71 ± 8.49	0.1587
eGFR (ml/min/1.73 m²)	85.97 ± 14.66	35.65 ± 6.4	<0.0001
BMI (kg/m²)	30.64 ± 5.54	28.88 ± 5.87	0.4229
Urine ACR (mg/g)	0.13 ± 0.16	1.79 ± 2.37	0.0093
Serum FGF23 (pg/ml)	80.92 ± 35.42	152 ± 34.81	<0.0001
Serum 25(OH)D (ng/ml)	11.6 ± 4	14.6 ± 5	0.0677
Serum 1,25(OH)$_2$D (pg/ml)	44 ± 19	43 ± 19	0.8329
Serum 24,25(OH)$_2$D (ng/ml)	1.24 ± 1	0.65 ± 0.5	0.0683
Serum PTH (pg/ml)	58 ± 28	167 ± 117	0.0007
Serum alkaline phosphatase (IU/L)	84.71 ± 25.71	95.57 ± 23.94	0.7304
Fractional excretion of calcium	0.69 ± 0.65	1.15 ± 1.85	0.2140
Fractional excretion of PO$_4$	12.26 ± 4.03	29.92 ± 8.63	<0.0001

Categorical variables are presented as percentage and continuous variables as mean ± SD. Fisher's exact test was used for the variable African American, Chi-square test for the variable DM. Unpaired t-test was used for the variables FGF-23, serum 25(OH)D, and BMI, Mann–Whitney U test was used for the rest of the continuous variables. eGFR, estimated glomerular filtration rate; BMI, body mass index; ACR, albumin creatinine ratio; FGF-23, fibroblast growth factor-23; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; 24,25(OH)$_2$D, 24,25-dihydroxyvitamin D; PTH, parathyroid hormone, PO$_4$; phosphorous.

Table 4

Univariate analysis of serum 24,25(OH)$_2$D.

Predictor variables	β	SE β	P value
Age (years)	−0.0054	0.00857	0.5285
BMI (kg/m^2)	−0.0016	0.01145	0.8899
Serum PTH (pg/ml)	−0.0018	0.00052	0.0016
Diabetes mellitus	0.09148	0.13541	0.5061
Serum FGF-23 (pg/ml)	−0.0029	0.00121	0.0225
Serum 25(OH)D (ng/ml)	0.02796	0.01237	0.0336
Serum 1,25(OH)$_2$D (pg/ml)	0.00416	0.0347	0.2437
Serum Ca (mg/dl)	0.37953	0.22884	0.1108
Serum PO$_4$ (mg/dl)	0.06079	0.09631	0.5344
Serum creatinine (mg/dl)	−0.1981	0.09307	0.0442
eGFR (ml/min/1.73 m^2)	−0.0390	0.00228	0.1009
Urine ACR(mg/g)	−0.0199	0.03495	0.5734
Fractional excretion of PO$_4$ (%)	−0.01204	0.00549	0.0392
Fractional excretion of Ca (%)	−0.02414	0.04732	0.6149

24,25(OH)$_2$D, 24,25-dihydroxyvitamin D; BMI, body mass index; PTH, parathyroid hormone; FGF-23, fibroblast growth factor-23; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; eGFR, estimated glomerular filtration rate; ACR, albumin creatinine ratio; PO4; phosphorous; Ca, calcium.

Table 5

Multivariable analysis of serum 24,25(OH)$_2$D

Predictor variables	β	SE β	P value
Serum PTH (pg/ml)	−0.0012	0.00049	0.0185
Serum FGF-23 (pg/ml)	−0.0023	0.00121	0.0349
Serum 25(OH)D (ng/ml)	0.03157	0.01237	0.0027

24,25(OH)$_2$D, 24,25-dihydroxyvitamin D; PTH, parathyroid hormone; FGF-23, fibroblast growth factor-23; 25(OH)D, 25-hydroxyvitamin D.