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Ferric citrate reduces fibroblast growth factor 23 levels and improves renal and cardiac function in a mouse model of chronic kidney disease

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Abstract

Iron deficiency, anemia, hyperphosphatemia, and increased fibroblast growth factor 23 (FGF23) are common and interrelated complications of chronic kidney disease (CKD) that are linked to CKD progression, cardiovascular disease and death. Ferric citrate is an oral phosphate binder that decreases dietary phosphate absorption and serum FGF23 concentrations while increasing iron stores and hemoglobin in patients with CKD. Here we compared the effects of ferric citrate administration versus a mineral sufficient control diet using the Col4a3 knockout mouse model of progressive CKD and age-matched wild-type mice. Ferric citrate was given to knockout mice for four weeks beginning at six weeks of age when they had overt CKD, or for six weeks beginning at four weeks of age when they had early CKD. Ten-week-old knockout mice on the control diet showed overt iron deficiency, anemia, hyperphosphatemia, increased serum FGF23, hypertension, decreased kidney function, and left ventricular systolic dysfunction. Ferric citrate rescued iron deficiency and anemia in knockout mice regardless of the timing of treatment initiation. Circulating levels and bone expression of FGF23 were reduced in knockout mice given ferric citrate with more pronounced reductions observed when ferric citrate was initiated in early CKD. Ferric citrate decreased serum phosphate only when it was initiated in early CKD. While ferric

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Author Contributions

VD and MW designed the study. CF, GC, CG, SN, XW, CD and LQ contributed to data acquisition. CF, GC, CG and VD analyzed the data and made the figures. VD, AM, MW, CF, GC, TI and RM contributed to data interpretation. CF, GC, VD and MW drafted the manuscript. All authors revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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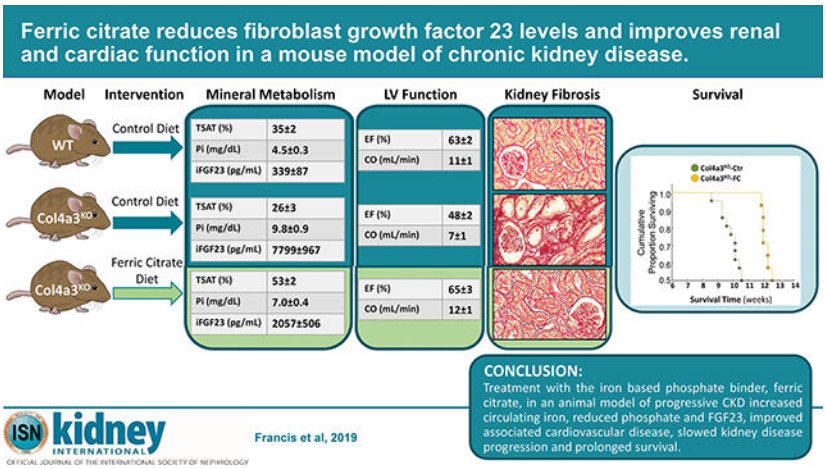
citrate mitigated systolic dysfunction in knockout mice regardless of timing of treatment initiation, early initiation of ferric citrate also reduced renal fibrosis and proteinuria, improved kidney function, and prolonged lifespan. Thus, initiation of ferric citrate treatment early in the course of murine CKD lowered FGF23, slowed CKD progression, improved cardiac function and significantly improved survival.

Graphical Abstract



Keywords

Anemia; Phosphate Binder; Chronic Kidney Disease; Phosphate; FGF23; Cardiac Disease

Introduction

FGF23 is an endocrine hormone that is secreted by osteocytes in response to high phosphate diets and states of impaired phosphate excretion, such as CKD. Under physiologic conditions, FGF23 stimulates phosphaturia and suppresses $1,25(OH)_2D$ levels, thereby maintaining normal phosphate homeostasis [1–3]. Although this compensatory response is initially adaptive, in the context of CKD, excess FGF23 leads to calcitriol deficiency that contributes to secondary hyperparathyroidism [3]. Excess FGF23 levels are also powerful predictors of CKD progression, cardiovascular disease and death, suggesting that in addition to its classical role in regulating bone and mineral metabolism, markedly elevated FGF23 levels in CKD may have additional effects that directly contribute to these adverse outcomes [4–10]. In support of this, FGF23 excess was shown to have a pathogenic role in development of cardiac hypertrophy [11–13] and dampening of host defenses in animal models of CKD [14, 15].

Ideal therapeutic approaches to reduce FGF23 in order to attenuate its potential adverse effects must lower FGF23 without sacrificing normal regulation of phosphate homeostasis because complete removal of FGF23 via administration of anti-FGF23 antibodies precipitates severe hyperphosphatemia that results in diffuse arterial calcification and early mortality [16]. However, current approaches to lower FGF23 by reducing phosphate, such as

dietary phosphate restriction or administering drugs that reduce gastrointestinal phosphate absorption, have had inconsistent effects in clinical trials [17, 18]. This underscores the need to develop novel approaches to reduce FGF23 in patients with CKD.

Disordered iron homeostasis and anemia are also nearly universal clinical features of progressive CKD that develop early in the course of CKD [19]. A link between iron metabolism and FGF23 regulation was first reported in 2011 in autosomal dominant hypophosphatemic rickets (ADHR) due to activating FGF23 mutations that impair its proteolytic cleavage [20–22]. In normal patients and mice, iron deficiency increases FGF23 production, but this was associated with increased proteolytic cleavage to yield FGF23 cleavage fragments that have not been shown to have biologic activity in bone and mineral metabolism [20–22]. In ADHR, iron deficiency increases circulating biologically active intact FGF23 (iFGF23) because the newly produced FGF23 cannot be cleaved. This results in hypophosphatemia, vitamin D deficiency, rickets and osteomalacia [20–22]. Notably, CKD appears to be analogous to ADHR in that it leads to a state of impaired FGF23 cleavage [23, 24]. We and others recently demonstrated that functional iron deficiency, inflammation, and erythropoietin all increase production of FGF23 and FGF23 cleavage, except in conditions that impair FGF23 cleavage, such as CKD, in which these stimuli increased biologically active iFGF23 [24–27].

Ferric citrate (Auryxia, Akebia Therapeutics Inc, Boston, MA, USA) was FDA-approved in the United States in 2014 for treatment of hyperphosphatemia in patients with CKD receiving dialysis and for the treatment of iron deficiency anemia (IDA) in CKD patients not yet on dialysis. Ferric citrate has shown promise in several studies to correct iron deficiency anemia and hyperphosphatemia and lower FGF23 levels in CKD patients [28–30]. Since ferric citrate is uniquely positioned among the phosphate binders to lower FGF23 levels through two independent mechanisms by simultaneously correcting iron deficiency and also binding to dietary phosphate, we tested the hypotheses that ferric citrate treatment will reduce FGF23 and mitigate negative outcomes in the Col4a3^{KO} mouse model of progressive CKD.

Results

Col4a3^{KO} mouse model of CKD

BUN and urinary albumin excretion were all significantly elevated in 9–10-week-old Col4a3^{KO} compared to wild-type (WT) mice fed standard chow (Fig1A–B). As previously published [31–33], circulating total FGF23, measured by a C-terminal assay (cFGF23), and intact FGF23 (iFGF23), were dramatically increased in Col4a3^{KO} compared to WT mice (Fig1C–D). Col4a3^{KO} mice also showed increased PTH and reductions in 1,25$(OH)_2$D together with increased serum phosphate and urinary phosphate excretion compared to WT (Fig1E–H). Systolic, diastolic and mean blood pressure (BP) were elevated in CKD mice compared to age-matched WT controls (Table 1). Finally, Col4a3^{KO} mice showed reduced levels of serum iron, transferrin saturation (TSAT), hemoglobin (Hb) and red blood cell (RBC) counts (Fig1I–L), demonstrating that iron absorption and transport are impaired and are accompanied by anemia of CKD.

Ferric citrate supplementation in mice with moderately advanced CKD reverses IDA

To investigate whether ferric citrate (FC) treatment can correct FGF23 elevations in Col4a3^{KO} mice, we first determined the optimal percentage of FC to be added to the food. We fed 8 week-old Col4a3^{KO} animals escalating doses of FC for one week (SFig1) and found that 5% FC supplementation is sufficient to reduce levels of cFGF23.

To further study the long-term impact of FC on FGF23 production, we conducted two different experimental protocols in which we treated Col4a3^{KO} mice with either early stage CKD (4-week-old mice) or overt CKD (6-week-old mice) a mineral sufficient control (Ctr) or a 5% FC diet versus age-matched WT mice. We collected all mice at 10 weeks of age for detailed investigation.

Six-week-old Col4a3^{KO} mice already show signs of impaired kidney function, evidenced by increased BUN and urine albumin excretion (Fig2A&B). Total and intact FGF23 were also slightly elevated in the Col4a3^{KO} mice (Fig2C&D). However, no changes were observed in phosphorus (Fig2E&F), 1,25(OH)$_2$D or PTH levels at this stage (data not shown). Circulating levels of iron, TSAT, Hb and RBC were similar in WT and Col4a3^{KO} mice, indicating that development of anemia occurs later in the course of CKD (Supplementary Table 1).

Col4a3^{KO} mice fed the control diet (Col4a3^{KO}-Ctr) had a significantly higher water intake and lower food intake (Fig3A–C) at the end of the study and consequently weighed less than diet matched WT mice (WT-Ctr). Four weeks of ferric citrate supplementation did not alter the water and food intake, and body weight was similar in FC compared to Ctr mice. As expected, FC supplementation increased fecal iron in both WT and CKD mice, compared to their respective controls (Fig3D).

Col4a3^{KO}-Ctr mice showed decreased serum iron and TSAT in parallel with decreased Hb and RBC (Fig3E–K). FC administration restored serum iron, TSAT and EPO and increased serum ferritin in Col4a3^{KO}-FC mice (Fig3E–G). As a likely consequence of increased circulating iron and increased EPO levels, Col4a3^{KO}-FC mice showed higher levels of Hb and RBC compared to Col4a3^{KO}-Ctr mice (Fig3J–K).

Effects of ferric citrate administration to mice with moderately advanced CKD on mineral metabolism

Despite the reduction in phosphate absorption, FC did not reduce circulating phosphate levels (Fig4A–B) and had no impact on serum calcium levels (SFig2). However, FC administration significantly reduced bone *Fgf23* expression, cFGF23 and iFGF23 levels in Col4a3^{KO} mice (Fig4C–E). The combined reduction of phosphate absorption and iFGF23 levels contributed to reduced urinary phosphate excretion in both WT and Col4a3^{KO} mice (Fig4F). FC supplementation increased 1,25(OH)$_2$D levels in WT and Col4a3^{KO} mice, but did not change PTH (Fig4G&H). In addition, FC supplementation did not correct the levels of BUN and albuminuria in Col4a3^{KO} mice (Fig4I&J). Finally, Col4a3^{KO}-Ctr mice displayed increased BP (Table2) and systolic dysfunction, as previously reported [33] and evidenced by reduced ejection fraction (EF), stroke volume and cardiac output (CO) (Fig4K–N). Interestingly, FC administration corrected BP and EF in Col4a3^{KO}-FC mice.

Taken together, these results suggest that FC administration to Col4a3^{KO} mice with moderately advanced CKD is able to prevent the dramatic increase in FGF23, hypertension and cardiac disease but does not delay CKD progression.

Effects of ferric citrate administration to mice with early CKD

Given the salutary effects of FC treatment on FGF23 and anemia in 6 week-old Col4a3^{KO} mice, we hypothesized that initiating FC in younger mice, before the onset of advanced CKD, could also have beneficial effects on kidney disease. To study the long-term impact of FC, we fed 4 week-old WT and Col4a3^{KO} mice, a mineral sufficient control (Ctr) or a 5% Ferric Citrate enriched (FC) diet for 6 weeks. As in the prior experiment, FC supplementation had no major effect on water and food intake in mice, although CKD animals fed a FC diet weighed less by the end of the experiment compared to age-matched untreated CKD mice (Fig5A–C). Likewise, FC increased iron absorption in both WT and Col4a3^{KO} mice, as evidenced by increased serum iron, TSAT, ferritin levels and liver iron content (Fig5E–L), and restored Hb levels in Col4a3^{KO} mice. Increased absorption of iron triggered an increase in duodenal iron retention in both WT and Col4a3^{KO} mice, shown by increased iron staining in duodenal microvilli (Fig5M). Of note, Col4a3^{KO}-Ctr mice display abnormally reduced expression levels of the iron transport genes, divalent metal transporter 1 (*Dmt1*) and transferrin receptor (*Tfrc*), indicating impaired iron absorption (Fig5N–P). Iron supplementation led to an increase in hepatic iron staining in both FC-fed WT and Col4a3^{KO} mice (Fig5Q), decreased inflammatory burden (Fig5R) coupled to a similar reduction in hepatic *Dmt1*, *Fpn* and *Tfrc* (Fig5S–U). Finally, FC diet increased hepatic expression of the iron regulatory protein hepcidin (*Hamp*), in WT-FC mice, and further increased Hamp expression in Col4a3^{KO}-FC mice (Fig5S–V).

Ferric citrate supplementation in early CKD reduces phosphate and FGF23 levels

FC supplementation significantly reduced phosphate absorption in both WT-FC and Col4a3^{KO}-FC mice, as shown by an increase in fecal phosphate and an increase in duodenal isoform of sodium-phosphate cotransporter solute carrier 34 A 2 (*Slc34a2, Npt2b*; Fig6A&B). The longer 6-week exposure to FC also reduced serum phosphate, and further reduced FGF23 bone expression and circulating levels in Col4a3^{KO}-FC mice (Fig6C–F), likely due to the combined increase in circulating iron levels and serum phosphate reduction. Reductions in serum FGF23 and phosphate contributed to a dramatic decrease in urinary phosphate excretion (Fig6G). FC administration restored 1,25$(OH)_2$D levels in Col4a3^{KO}-FC mice, likely due to FGF23 reduction, but did not modify CKD associated hyperparathyroidism (Fig6H–I). Increased renal FGF23 signaling in CKD is evidenced by increased mRNA expression of early growth response protein 1 (*Egr1*) in Col4a3^{KO}-Ctr mice, suppression of the vitamin D anabolic enzyme (*Cyp27b1*) and stimulation of vitamin D catabolic enzyme (*Cyp24a1*) (Fig6J–L). In line with our findings, FC supplementation reduced *Egr1* in Col4a3^{KO}-FC mice, and restored *Cyp27b1* and *Cyp24a1* expression.

Ferric citrate supplementation in early CKD delays CKD progression and prevents hypertension

In association with improved iron status and reduced circulating phosphate and FGF23 levels, Col4a3^{KO}-FC mice unexpectedly showed improved kidney function after 6 weeks of

FC supplementation, evidenced by reduced BUN and albuminuria (Fig7A&B), suggesting that early delivery of FC slowed progression of kidney disease. Reductions in interstitial fibrosis and tubular atrophy were also evident by histology in Col4a3^{KO}-FC animals compared to Col4a3^{KO}-Ctr group (Fig7C). FC administration significantly reduced kidney expression of several collagen isoforms *Col1a1*, *Col3a1* and *Col6a1* in Col4a3^{KO}-FC mice (Fig7D–F), which are associated with renal fibrosis and were highly elevated in Col4a3^{KO}-Ctr mice. In addition, administration of FC to Col4a3^{KO} mice corrected the increase in BP observed in Col4a3^{KO}-Ctr mice (Fig7G–H). Overall, Col4a3^{KO}-FC mice exhibited attenuated activation of MEK/ERK and PLCγ/calcineurin/NFAT pathways, two major signaling pathways for FGF23 (Fig7J). To test the immediate effects of FGF23 on the activation of these pathways, 10 week old WT mice received a single bolus of 100 μL of either 0.9% sodium chloride (Ctr), 25 μg/g ferric citrate (FC), 50 μg/g of sodium monobasic phosphate (Pi; Sigma-Aldrich, MO, USA) or 50 ng/g of FGF23 (R&D Systems, MN, USA). Both phosphate and FGF23 induced MEK/ERK phosphorylation, while only FGF23 induced activation of PLCγ/calcineurin/NFAT, suggesting that a reduction of MEK/ERK in CKD is likely due to a reduction of both phosphate and FGF23 burden, while decreased FGF23 explains the reduction in PLCγ/calcineurin/NFAT activity (Fig7K). Further analysis showed that FGF23, and to a much lower extent iron, increases *Egr1* expression and *Nfat4* expression (Fig7L–P). Finally, phosphate but not FGF23 or iron appears to induce collagen expression (Fig7Q–S), while only FGF23 reduces *Cyp27b1* and increases *Cyp24a1* expression (Fig7T&U).

Ferric citrate supplementation in early CKD rescues systolic dysfunction

Compared to WT-Ctr mice, EF, stroke volume and CO were significantly lower in Col4a3^{KO}-Ctr mice consistent with the development of systolic dysfunction (Fig8A–D). These alterations in cardiac function were significantly attenuated by FC in Col4a3^{KO}-FC mice (Fig8A–D). In line with previous findings[13, 33], expression of markers of hypertrophy were reduced (B-type natriuretic peptide -*Bnp,* Atrial natriuretic peptide-*Anp*, β-Myosin Heavy Chain- *Myh7*) in Col4a3^{KO}-Ctr mice (Fig8E–G). Expression of *Fgfr4*, which mediates the hypertrophic effects of FGF23 on cardiac myocytes [12], was significantly reduced compared to WT-Ctr mice, whereas expression of *Fgfr3* was significantly increased and paralleled increased *Egr1* expression (Fig8H–L). FC further reduced *Fgfr4* expression in Col4a3^{KO}-FC mice but attenuated the increase of *Fgfr3* and *Egr1*. Further analysis of MEK/ERK and PLCγ/calcineurin/NFAT pathways in the heart showed increased activity in Col4a3^{KO}-Ctr mice compared to WT-Ctr mice, and a reduction in Col4a3^{KO}-FC mice (Fig8M), perhaps due to reduced FGF23 signaling. Acute administration of phosphate and FGF23 to WT mice inducde MEK/ERK phosphorylation, while only FGF23 activates PLCγ/calcineurin/NFAT. Thus, reduced MEK/ERK signaling in Col4a3^{KO}-FC mice is mainly due to reduced phosphate and FGF23, while reduced PLCγ/calcineurin/NFAT signaling might be attributed to decreased circulating FGF23 (Fig8N). Of note, phosphate administration reduces phosphorylation of PLC gamma. Iron and phosphate both increase the expression of *Fgfr3*, suggesting that higher levels of phosphate in CKD might increase FGF23 signaling through FGFR3. Acute administration of FGF23 paradoxically raises the expression of *Fgfr4* and *Egr1* expression in the heart (Fig8O–S). In line with our previous results FGF23, but not phosphate increases the expression of *Nfat4* (Fig8T–W). Finally, as a

likely consequence of the improvements in the renal and cardiac phenotypes following FC administration, Col4a3^{KO}-FC mice lived an average of 2 weeks longer than Col4a3^{KO}-Ctr mice, corresponding to a significant 20% extension of lifespan (Fig 8M).

Discussion

Hyperphosphatemia, anemia and excess FGF23 are associated with adverse outcomes in patients with CKD [34–36]. Ferric citrate, an iron-based phosphate binder, has shown promise in clinical studies to reduce phosphate absorption and FGF23 levels, and to replete iron stores and ameliorate anemia in CKD [28, 37–40]. In two separate experiments using the Col4a3^{KO} mouse model of progressive CKD, we show that administration of ferric citrate to animals with early or more advanced CKD decreases intestinal phosphate absorption, increases iron stores, corrects anemia and reduces FGF23 levels. Surprisingly, FC intervention beginning in animals with early CKD preserved renal function and mitigated the development of albuminuria and tubulointerstitial fibrosis. In addition, FC improved cardiac function of CKD mice in both experiments independently of its effects on CKD progression. Finally, improvement in mineral homeostasis and heart and kidney dysfunction significantly extended the lifespan of Col4a3^{KO} mice.

Animals tolerated the relatively high dose of iron and showed no overt symptoms of iron overload in terms of activity or behavior, consistent with a low potential for iron overload by oral absorption [41] and even lower in patients with CKD who have high levels of hepcidin that downregulates intestinal iron absorption [42]. Ferric citrate repleted iron stores and rescued anemia in CKD animals, as in prior human studies [43], independently of treatment duration despite a significant increase in hepatic hepcidin expression. It is unlikely that the increases in serum ferritin were related to increased inflammatory burden, because hepatic expression of *Saa1*, an inflammatory marker, was reduced, while TSAT and hepatic iron increased.

Our findings also show that oral ferric citrate performed as expected by reducing enteric phosphate absorption independently of treatment duration. In contrast to the 4-week FC diet, reduction of enteric phosphate reabsorption in early CKD animals supplemented with FC for 6 weeks led to a significant decrease in circulating phosphate levels. Overall, these findings suggest potential benefits of earlier intervention to reduce phosphate levels in CKD, even in the absence of overt hyperphosphatemia. Hyperphosphatemic Col4a3^{KO}-Ctr also showed high duodenal expression of the sodium phosphate co-transporter Npt2b which mediates the active transport component of intestinal phosphate absorption. This suggests that the duodenal phosphate transport may be pathologically altered in the setting of CKD, but further studies are required.

As a likely consequence of increased iron and decreased phosphate absorption, FGF23 levels were significantly decreased following FC administration, similar to previous findings [30, 38, 39]. Although FC treatment had different effects on serum phosphate and anemia depending on its timing of initiation and duration, FC reduced osseous *Fgf23* transcription in Col4a3^{KO} animals by ~3 fold, regardless of diet duration. Both the 4-week and 6-week administrations were successful in reducing circulating cFGF23 and iFGF23, with a more

pronounced effect in the 6-week administration. Importantly, FC administration decreases FGF23 production in the 4-week study without reducing circulating phosphate levels in Col4a3^{KO}-FC mice. This suggests that phosphate absorption rather than circulating phosphate controls FGF23 production, similar to prior findings [44].

Decreased phosphate absorption and reductions in iFGF23 resulted in a dramatic decrease in urine phosphate excretion in Col4a3^{KO}-FC animals, which was reduced to the level of WT-Ctr despite ~3000pg/ml serum iFGF23, hyperphosphatemia, and hyperparathyroidism. A differential response in PTH to ferric citrate administration was also noted in clinical studies, in which reductions of FGF23 resulted in elevation of intact PTH levels in hemodialysis patients receiving ferric citrate [30], while PTH levels significantly decreased compared with baseline following ferric citrate treatment in CKD patients whereas FGF23 levels remained unchanged [38]. Our findings are also consistent with previous studies describing lack of changes of PTH, and persistent downregulation of intact FGF23 upon inducible and global removal of *Slc34a2*, resulting in decreased intestinal phosphate absorption [45]. This remarkable persistence of hyperparathyroidism in Col4a3^{KO}-FC animals is even more intriguing given the modest but significant rescue of 1,25$(OH)_2$D levels, likely due to iFGF23 reductions. This suggests that in addition to FGF23 and 1,25$(OH)_2$D other mechanisms control PTH secretion in CKD.

Our results indicate that administration of ferric citrate has benefits extending beyond its effects on mineral metabolism. FC administration to animals with early CKD considerably improved kidney morphology and function. Although the pathogenesis of kidney injury is likely multifactorial, the duration of exposure to phosphate and FGF23 might affect the rate of the decline in renal function. Under physiological conditions, the intestinal absorption of phosphate is matched by an equivalent urinary excretion; a recent report showed that phosphaturia in patients with normal serum phosphate predicts renal disease progression [46]. Col4a3^{KO} mice show increased phosphate absorption, likely due to increased duodenal *Slc34a2* mRNA, overt hyperphosphatemia and hyperphosphaturia, due to increased phosphaturic hormones FGF23 and PTH. Our data suggests that by directly reducing phosphate absorption and decreasing FGF23, leading to reduced phosphaturia, FC could attenuate kidney disease progression. Consistent with a potential pro-fibrotic effect of phosphate and FGF23 [47], Col4a3^{KO}-Ctr mice showed increased renal fibrosis that was reduced by FC along with parallel decreases in *Col1a1*, *Col3a1*, and *Col6a1* mRNA expression. In addition, we further showed that acute administration of phosphate alone induces the expression of *Col1a1* and *Col6a1* in WT mice.

Administration of FC improved the cardiac phenotype of CKD mice independently of kidney function, since an improvement in cardiac function was observed in both studies, even in mice treated with FC from 6 to 10 weeks, in absence of measurable improvement in kidney function in Col4a3^{KO}-FC mice. Col4a3^{KO}-Ctr mice showed hypertension, reduced EF and stroke volume indicative of systolic dysfunction, but they did not develop LVH [33], which is the major cardiovascular phenotype associated with FGF23 excess in clinical and animal studies [5, 11, 12]. Col4a3^{KO} mice also show reduced markers of cardiac hypertrophy and decreased FGFR4. Thus, improvement in cardiac function following FC administration is unlikely due to reductions in FGF23-FGFR4 signaling. Although it is extremely

challenging to dissociate the effects of phosphate, iron and FGF23 corrections on the heart, this suggests that reduction in FGF23, independently of kidney disease progression and circulating phosphate levels, might be associated with improvement of cardiac function. In this case, FGF23 could signal through other FGFR isoforms [48], including FGFR3 which is elevated in Col4a3^{KO} mice [49], perhaps due to hyperphosphatemia. FGF23 activation of Raf/MEK/ERK pathway and/or *Egr1* expression [49, 50] might induce systolic dysfunction, which is another clinical complication of CKD [51]. Indeed, selective inhibition of *Egr1* prevents the onset of systolic dysfunction [52]. Consistent with this hypothesis, Col4a3^{KO}-FC mice show decreased Egr1 compared to Col4a3^{KO}-Ctr mice and MEK/ERK activation. Thus, this might represent another mechanism of FGF23-induced cardiac injury in CKD. Nonetheless, despite the reduction of *Fgfr4* expression, PLCγ/calcineurin/NFAT activity is increased in CKD mice and attenuated by FC treatment suggesting that FGF23 may also signal through this pathway [11, 12].

Alternatively, the rescue of the cardiac phenotype in Col4a3^{KO}-FC animals might be directly related to increased circulating iron and rescue of anemia, consistent with prior reports showing increased ejection fraction following iv iron administration [53, 54]. Indeed, the heart is particularly susceptible to iron deficiency and anemia. The myocardium has a high demand in oxygen [55], thus reduced hemoglobin adversely impacts cardiac function. The heart also relies on free fatty acids as a primary energy source and restricted iron limits the synthesis of iron-sulfur proteins involved in oxidative phosphorylation and ATP generation.

Although further studies testing the effects of FC in other models of CKD are needed to fully establish the beneficial effects of ferric citrate on kidney and cardiac functions, exploratory path analysis (SFig3) of changes in BUN partially mediated by changes in TSAT, and indirect additional significant effects of ferric citrate treatment. In addition, path analyses of changes of cardiac output, highlighted significant partial mediation of improvement in cardiac function by changes in iFGF23, TSAT and serum phosphate and additional indirect effects of ferric citrate treatment.

In aggregate, our results suggest that the combined corrections of FGF23 and phosphate excess and increases in iron stores following FC administration might improve the kidney and cardiovascular diseases in patients with CKD.

Materials and Methods

Animals

Heterozygous 129X1/SvJ-Col4a3^{tm1Dec} mice were crossed to generate 129X1/SvJ-Col4a3$^{+/+}$ (WT) and 129X1/SvJ-Col4a3$^{-/-}$ (Col4a3^{KO}). Mice were maintained on a standard diet (Teklad 7012, Harlan Teklad, USA) containing 240 ppm iron, except when otherwise specified. To assess the effects of ferric citrate (FC), 4 week-old mice or 6 week-old mice were fed a mineral adequate control diet that contained 48 ppm of iron, enriched or not with 5% ferric citrate. In a separate set of animals, we recorded the age of death on ten Col4a3^{KO} male littermates per group to assess effects on lifespan. Finally, 10 week-old WT mice received a single bolus of 100 μL of either 0.9% sodium chloride (Ctr), 25 μg/g ferric citrate (FC), 50 μg/g of sodium monobasic phosphate (Pi; Sigma-Aldrich, MO, USA) or 50 ng/g of

FGF23 (R&D Systems, MN, USA). Animals were collected two hours post-injection. All experiments were performed in male mice. All studies were approved by Institutional Animal Care and Use Committee at Northwestern University.

Biochemistry

We collected overnight urine samples from fasted animals housed overnight in metabolic cages and serum samples by intracardiac exsanguination. We used a murine intact FGF23 ELISA assay to measure the iFGF23 and a cFGF23 ELISA to measure total FGF23 (Quidel, Carlsbad, CA, USA). We measured parathyroid hormone (PTH) using a mouse intact ELISA (Quidel), 1,25-dihydroxyvitamin D [1,25$(OH)_2$ D] by immunoassay (Immundiagnostik Inc., Manchester, NH, USA) and calcium, phosphate, blood urea nitrogen (BUN), albumin, creatinine, iron and transferrin saturation (TSAT) using colorimetric assays (Pointe Scientific, Canton, MI, USA). Serum ferritin and transferrin levels were measure using mouse ELISA assays (Alpco, Salem, NH, USA).

RT-PCR

Total RNA was isolated using TRI-reagent, and first-strand cDNA was synthesized from different organs [56]. The iCycler iQ real-time PCR detection system and iQ SYBR Green supermix (Bio-Rad Laboratories, USA) were used for real-time quantitative PCR analysis. The expression was normalized to glyceraldehyde-3-phosphate dehydrogenase in the same sample and expressed as fold-change versus wild-type.

Blood pressure

We measured blood pressure (BP) in sentient mice as previously described[13, 33].

Echocardiography

We performed echocardiography under isoflurane anesthesia 1 week prior to sacrifice (at 9 weeks of age) as previously described [13, 33].

Hematologic analysis

Hematologic parameters were acquired in whole blood using the HEMAVET 950 hematology system (Drew Scientific Inc., Oxford, CT, USA) and analyzed with multispecies software using mouse settings.

Histology

Duodena, kidneys and livers were collected at sacrifice, fixed in 100% ethanol and embedded in paraffin. We collected 5-μm-thick sections using a rotary microtome. We stained the sections with hematoxylin and eosin (H&E) to determine duodenal, renal and hepatic morphology, picrosirius red (PSR) to determine renal fibrosis, and Perls' prussian blue for iron content. Images were acquired using bright field microscopy (Leica Microsystems, Buffalo Grove, IL, USA.

SDS-Page

Tissue lysates were prepared in using T-Per lysis buffer (Thermo Fisher Scientific, MA, USA) containing protease inhibitors cocktail and immunoblots performed as previously described [24] using rabbit antibodies against phosphorylated Erk1/2 (p44/42 MAPK) (1:1000; 4370S; Cell Signaling), Erk1/2 (p44/42 MAPK) (1:1000; 4659S), phosphorylated MEK1/2 (1:1000; 9154S), MEK1/2 (1:1000; 9122L), phosphorylated phospholipase C gamma 1 (1:1000; 8713S), pan calcineurin (1:1000, 2614S), NFAT4 (1:1000, 4998S) from Cell Signaling (MA, USA) and rabbit anti-phospholipase C gamma 1 (1:1000; 76155) and goat anti-β Actin (1:1000, 8229) from abcam (MA, USA).

Statistics

Data are presented as mean ± SE. ANOVA followed by Fisher and *t* tests were used for statistical difference using Statistica software (Statsoft, OK, USA). P values < 0.05 were considered statistically significant. To test the major effects of ferric citrate treatment we used structural equation modeling (path analysis) using SEPATH module (SFig3). Generalized least square followed by maximum likelihood was used as discrepancy function to validate our model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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MW has received consulting fees from Keryx, Akebia, Amgen, Diasorin, Ardelyx, and Pharmacosmos.

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Translational Statement

Our study performed in an animal model of progressive CKD suggests that patients with CKD may benefit from a combined reduction of dietary phosphate intake and increased serum iron. In mice with CKD, early treatment with the iron based phosphate binder, ferric citrate, reduced the magnitude of FGF23 elevation, attenuated associated cardiovascular disease, slowed kidney disease progression, and prolonged survival. This suggests that ferric citrate might mitigate cardiac and renal injury and possibly improve survival in patients with CKD.

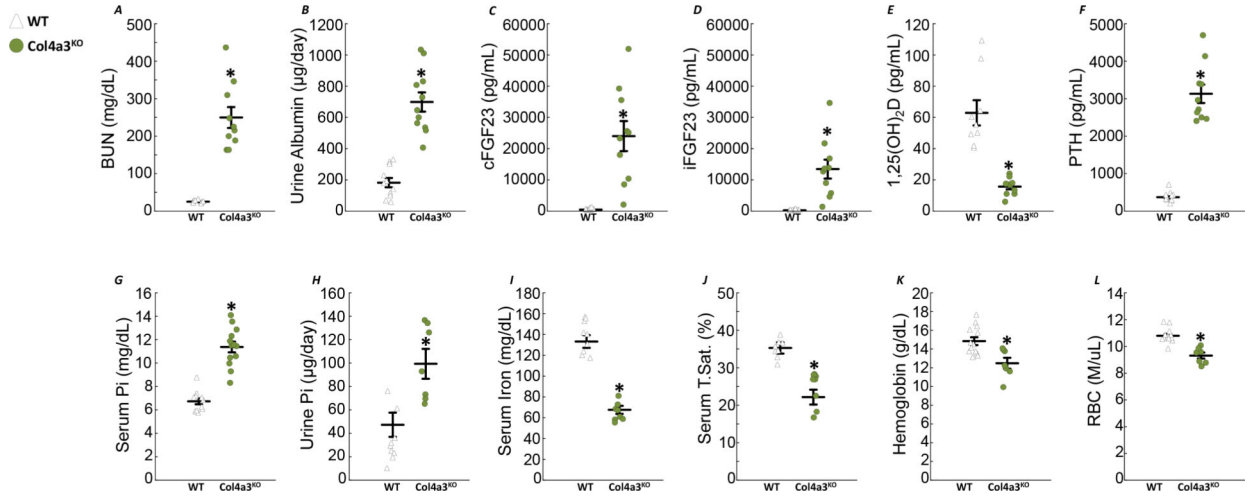

Figure 1: Col4a3^{KO} mice: a model of chronic kidney disease with anemia

Levels of serum BUN (A), urine albumin (B), serum cFGF23 (C), serum iFGF23 (D), serum 1,25(OH)$_2$D (E), serum PTH (F), serum phosphate (G), urine phosphate (H), serum iron (I), serum transferrin saturation (J), hemoglobin (K) and red blood cell number (L) in 9–10 week old WT and Col4a3^{KO} mice fed a standard diet. Data are presented as mean ± SE, n≥11 per group, $p<0.05$ vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice.

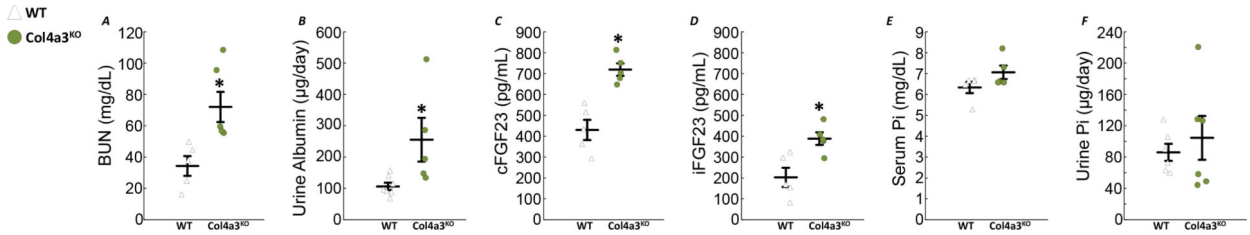

Figure 2: FGF23 increase is an early event during CKD progression

Levels of serum BUN (A), urine albumin (B), serum cFGF23 (C), serum iFGF23 (D), serum phosphate (E), urine phosphate (F) in 6 week old WT and Col4a3^{KO} mice fed a standard diet. Data are presented as mean ± SE, n≥11 per group, $p<0.05$ vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice.

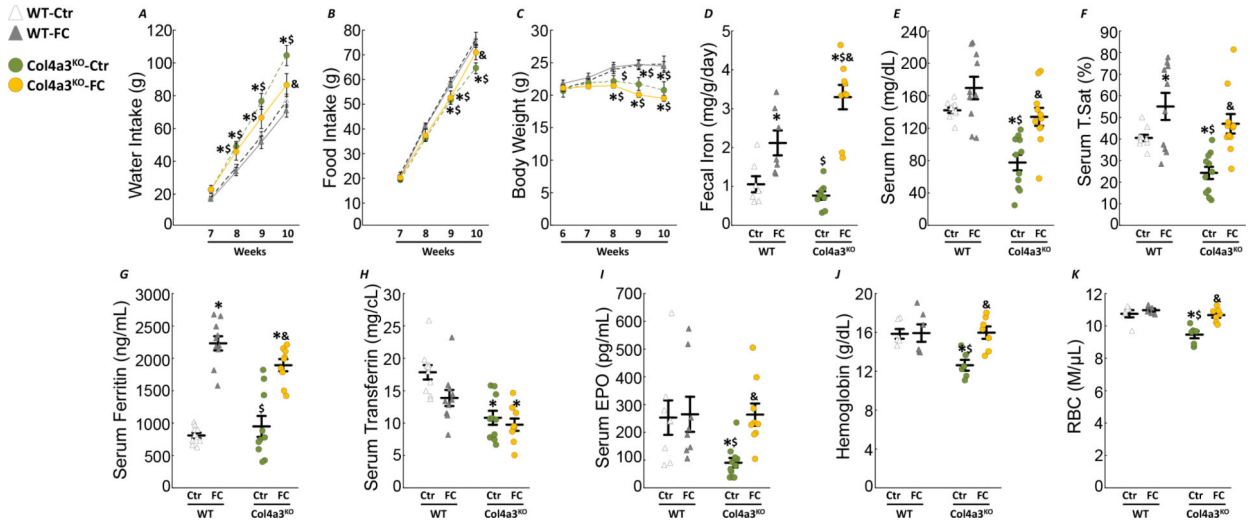

Figure 3: Ferric citrate administration in mice with moderate CKD improves iron deficiency

Longitudinal measurements of cumulative (A) water intake, (B) food intake and body weight (C). Levels of fecal iron (D), serum iron (E), serum transferrin saturation (F), serum ferritin (G), serum transferrin (H), serum erythropoietin (I), hemoglobin (J) and red blood cell number (K) in 10 week old WT and Col4a3^{KO} mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 4 weeks. Data are presented as mean ± SE, n≥8 per group, p<0.05 vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice.

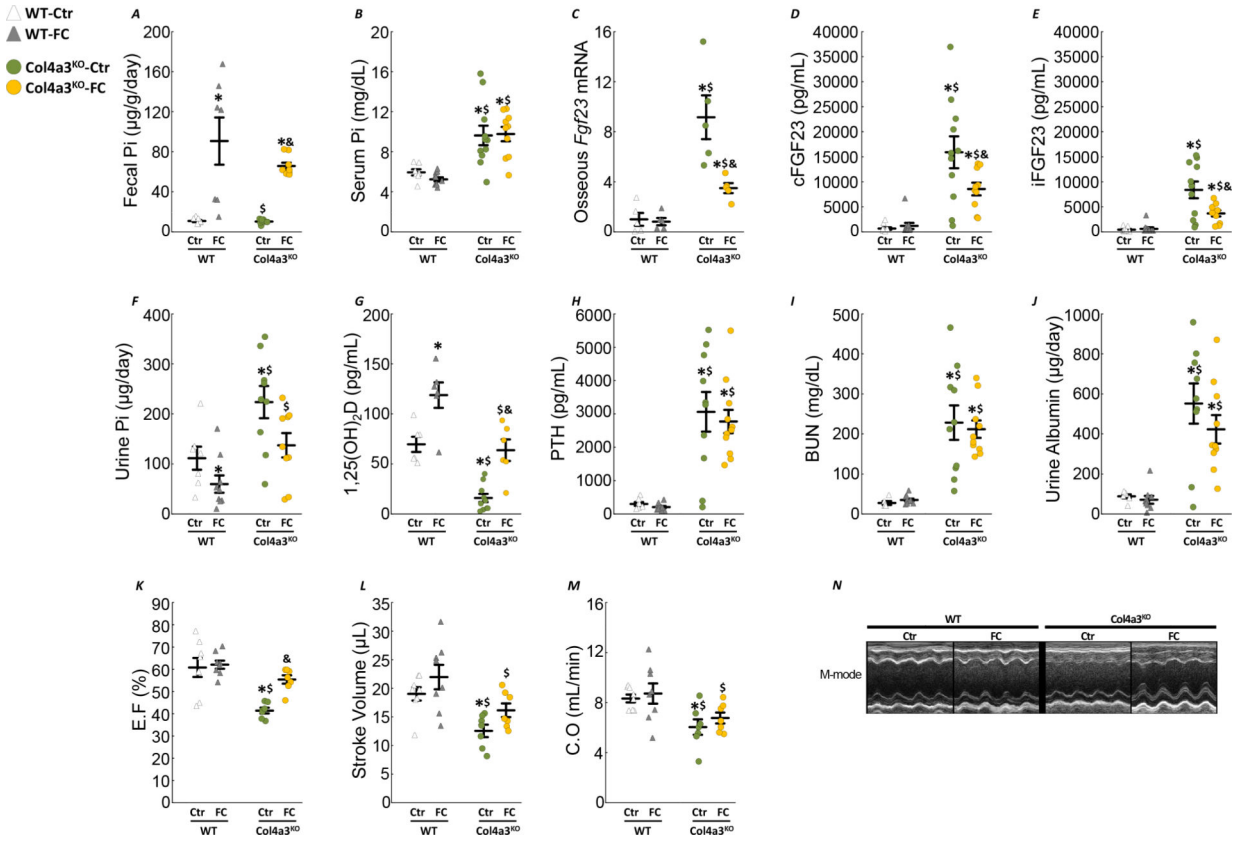

Figure 4: Ferric citrate administration in mice with moderate CKD reduces FGF23 production

Levels of fecal phosphate (A), serum phosphate (B), bone *Fgf23* expression (C), serum cFGF23 (D), serum iFGF23 (E), urine phosphate (F), serum $1,25(OH)_2D$ (G), serum PTH (H), serum BUN (I) and urine albumin (J). Echocardiography analysis of left ventricular ejection fraction (K), stroke volume (L), cardiac output (M) and representative M-mode echocardiography (N). Measurements performed in 10 week old WT and $Col4a3^{KO}$ mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 4 weeks. Data are presented as mean ± SE, n≥8 per group, $p<0.05$ vs.* WT-Ctr, $ WT-FC, & $Col4a3^{KO}$-Ctr mice.

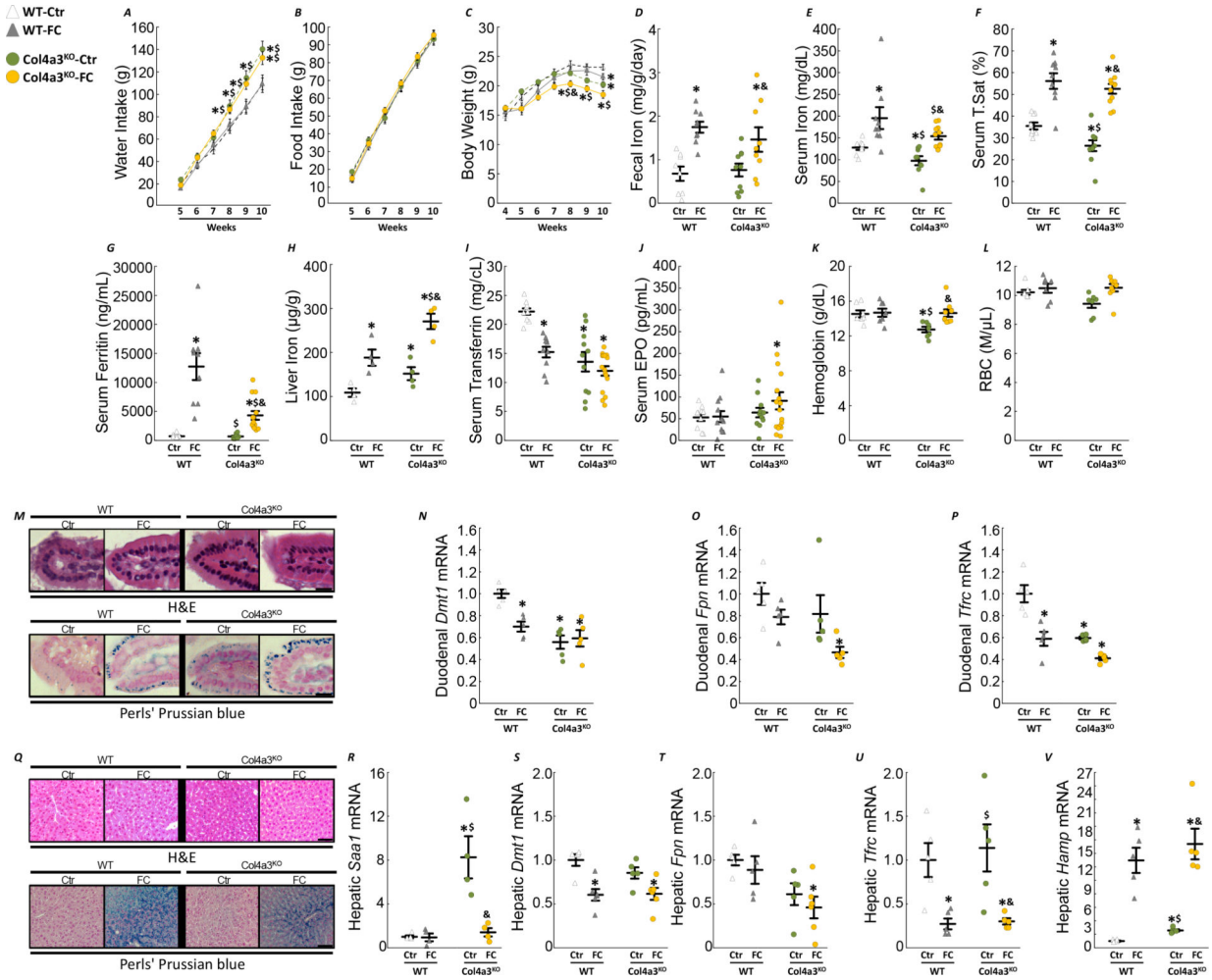

Figure 5: Ferric citrate administration in mice with early CKD corrects anemia of CKD.

Longitudinal measurements of cumulative (A) water intake, (B) food intake and body weight (C). Levels of fecal iron (D), serum iron (E), serum transferrin saturation (F), serum ferritin (G), liver iron (H), serum transferrin (I), serum erythropoietin (J), hemoglobin (K) and red blood cell number (L) in 10 week old WT and Col4a3^{KO} mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 6 weeks. Data are presented as mean ± SE, n≥8 per group. Representative sections of duodenal microvilli stained with hematoxylin & eosin or Perls' Prussian blue iron staining (M, bar=15 µm) and mRNA expression of markers of iron metabolism in duodenum, *Dmt1* (N), *Fpn* (O) and *Tfrc* (P). Representative sections of liver stained with hematoxylin & eosin or Perls' Prussian blue iron staining (Q, bar=50 µm) and hepatic mRNA expression of inflammatory marker *Saa1* (R) and of markers of iron metabolism, *Dmt1* (S), *Fpn* (T), *Tfrc* (U) and *Hamp* (V). Data are presented as mean ± SE, n≥4 per group, p<0.05 vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice. Measurements performed in 10 week old WT and Col4a3^{KO} mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 6 weeks.

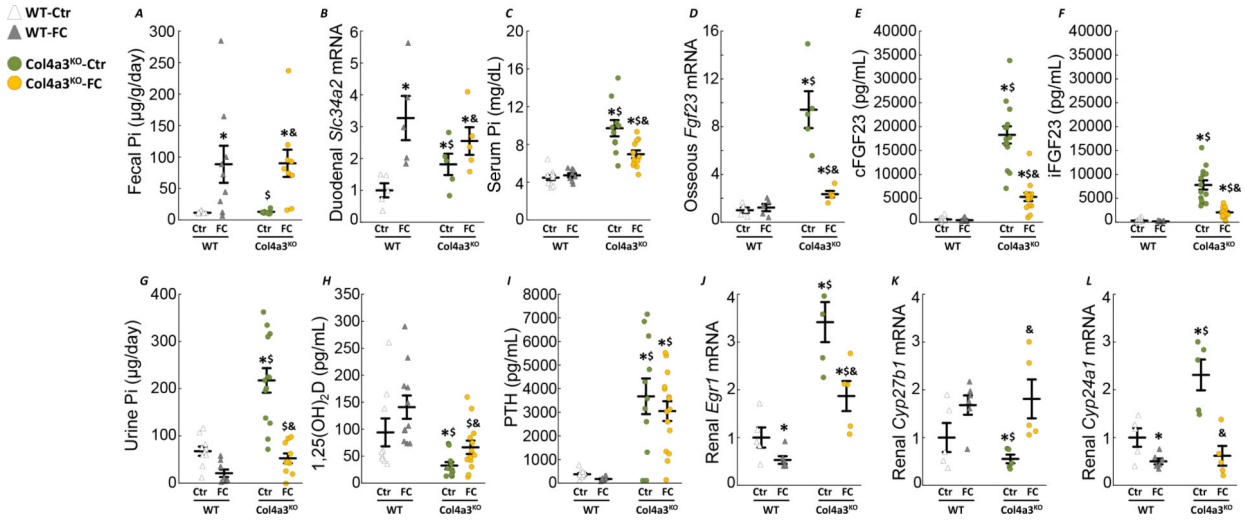

Figure 6: Ferric citrate administration in mice with early CKD decreases FGF23 production.

Levels of fecal phosphate (A), duodenal slc34a2 (B), serum phosphate (C), osseous Fgf23 mRNA expression (D), serum cFGF23 (E), serum iFGF23 (F), urine phosphate (G), serum $1,25(OH)_2D$ (H) and serum PTH (I) in 10 week old WT and Col4a3^{KO} mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 6 weeks. Data are presented as mean ± SE, n≥8 per group, $p<0.05$ vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice. Renal mRNA expression of FGF23 targets, *Egr1* (J), *Cyp27b1* (K), *Cyp24a1* (L). Data are presented as mean ± SE, n≥5 per group, $p<0.05$ vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice. Measurements performed in 10 week old WT and Col4a3^{KO} mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 6 weeks.

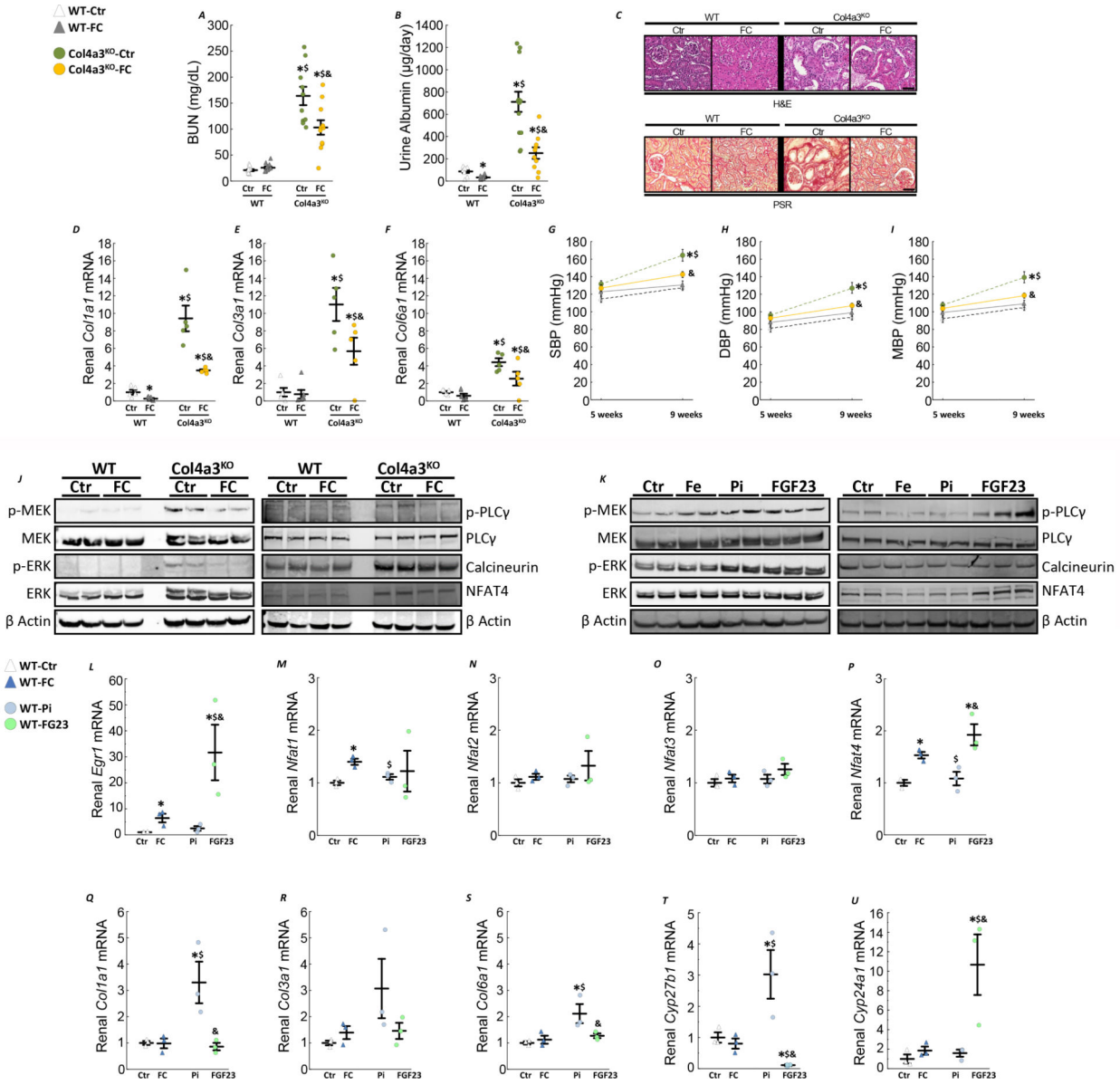

Figure 7: Ferric citrate administration in mice with early CKD slows CKD progression.

Levels of serum BUN (A), urine albumin (B) in 10 week old WT and Col4a3^{KO} mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 6 weeks. Data are presented as mean ± SE, n≥8 per group, p<0.05 vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice. Representative sections of kidney stained with hematoxylin & eosin or picro-sirius red staining (C, bar=50 µm) and renal mRNA expression of markers of fibrosis, *Col1a1* (D), *Col3a1* (E) and *Col6a1* (F). Measurements performed in 10 week old WT and Col4a3^{KO} mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 6 weeks. Data are presented as mean ± SE, n≥5 per group, p<0.05 vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice. Non-invasive systolic (G), diastolic (H) and mean blood pressure (I). Data are presented as mean ± SE, n≥11 per group, p<0.05 vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice. (J) Representative immunoblots of kidney protein extracts

stained for MEK/ERK and PLCγ/calcineurin/NFAT pathways activity. (K) Representative immunoblots of kidney protein extracts for MEK/ERK and PLCγ/calcineurin/NFAT pathways from 10 week old WT mice collected two hours post-injection of either 0.9% sodium chloride (Ctr), 25 μg/g ferric citrate (FC), 50 μg/g of sodium monobasic phosphate (Pi) or 50 ng/g of FGF23. Renal mRNA expression of FGF23 potential targets, *Egr1* (L), *Nfat1* (M), *Nfat2* (N), *Nfat3* (O), *Nfat4* (P), markers of fibrosis, *Col1a1* (Q), *Col3a1* (R) and *Col6a1* (S) and vitamin D enzymes *Cyp27b1* (T), *Cyp24a1* (T). Data are presented as mean ± SE, n≥3 per group, $p<0.05$ vs.* WT-Ctr, $ WT-FC, & WT-Pi mice.

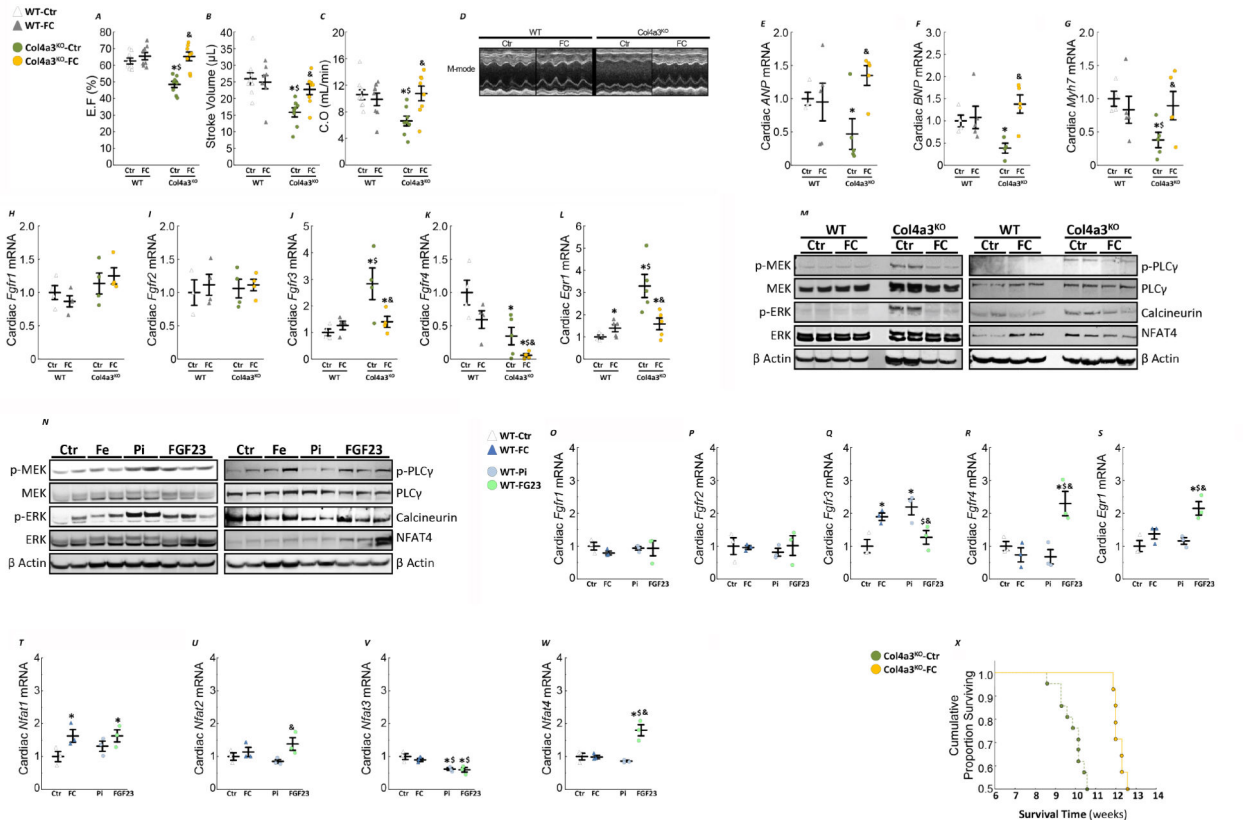

Figure 8: Ferric citrate administration in mice with early CKD improves cardiac outcomes and survival.

Echocardiography analysis of left ventricular ejection fraction (A), stroke volume (B) and cardiac output (C) and M-mode echocardiography (D). Data are presented as mean ± SE, n≥8 per group, p<0.05 vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice. mRNA expression of cardiac markers of hypertrophy *ANP* (E), *BNP* (F) and *Myh7* (G)and FGF23 signaling *Fgfr1* (H), *Fgfr2* (I), *Fgfr3* (J), *Fgfr4* (K), *Egr1* (L). Data are presented as mean ± SE, n≥3 per group, p<0.05 vs.* WT-Ctr, $ WT-FC, & Col4a3^{KO}-Ctr mice. (M) Representative immunoblots of heart protein extracts stained for MEK/ERK and PLCγ/calcineurin/NFAT pathways activity. Measurements performed in 10 week old mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) during 6 weeks. (N) Representative immunoblots of heart protein extracts for MEK/ERK and PLCγ/calcineurin/NFAT pathways from 10 week old WT mice collected two hours post-injection of either 0.9% sodium chloride (Ctr), 25 µg/g ferric citrate (FC), 50 µg/g of sodium monobasic phosphate (Pi) or 50 ng/g of FGF23. mRNA expression of cardiac markers FGF23 signaling *Fgfr1* (O), *Fgfr2* (P), *Fgfr3* (Q), *Fgfr4* (R), *Egr1* (S), *Nfat1* (T), *Nfat2* (U), *Nfat3* (V), *Nfat4* (W). Data are presented as mean ± SE, n≥3 per group, p<0.05 vs.* WT-Ctr, $ WT-FC, & WT-Pi mice. (X) Cumulative proportion surviving of Col4a3^{KO} mice fed a mineral sufficient diet (Ctr) supplemented or not with 5% ferric citrate (FC) starting at 4 weeks of age.

Table 1:

Elevated BP in Col4a3^{KO} mice.

	WT	Col4a3^{KO}
Systolic BP (mmHg)	139±3	170±7 *
Diastolic BP (mmHg)	100±3	123±6 *
Mean BP (mmHg)	112±3	138±6 *

Non-invasive systolic, diastolic and mean blood pressure (BP). Values are Mean ± SE, n≥13 per group

* $p<0.05$ vs. WT.

Table 2:

Administration of ferric citrate corrects blood pressure increase in Col4a3^{KO} mice with moderate CKD.

	WT		Col4a3^{KO}	
	Ctr	**FC**	**Ctr**	**FC**
Systolic BP (mmHg)	142±5	145±4	165±6 *$	150±4 &
Diastolic BP (mmHg)	104±3	106±4	124±6 *$	108±5 &
Mean BP (mmHg)	117±3	119±4	137±6 *$	122±4 &

Non-invasive systolic, diastolic and mean blood pressure (BP) in 10-week old animals. Values are Mean ± SE, n≥11 per group, $p<0.05$ vs.

*WT-Ctr

$WT-FC

&Col4a3^{KO}-Ctr mice.