# Tumor necrosis factor stimulates fibroblast growth factor 23 levels in chronic kidney disease and non-renal inflammation

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# Antibody mediated TNF neutralization decreases FGF23 levels in animal models of chronic kidney disease and non-renal inflammation

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# Abstract

Fibroblast growth factor 23 (FGF23) regulates phosphate homeostasis and its early rise in patients with chronic kidney disease (CKD) is independently associated with all-cause mortality. Since inflammation is characteristic for CKD and has been associated with plasma FGF23 we examined whether inflammation directly stimulates FGF23. In a population-based cohort, plasma tumor necrosis factor (TNF) was the only inflammatory cytokine that independently and positively correlated with plasma FGF23. Mouse models of CKD showed signs of renal inflammation, renal FGF23 expression and elevated systemic FGF23. Renal FGF23 expression coincided with expression of the orphan nuclear receptor Nurr1 regulating FGF23 in other organs. Antibody-mediated neutralization of TNF normalized plasma FGF23 and ectopic renal *Fgf23* expression. Conversely, TNF administration to control mice increased plasma FGF23 without altering plasma phosphate. Similarly, in *Il10* deficient mice with inflammatory bowel disease and normal kidney function, FGF23 was elevated and normalized upon TNF neutralization. In conclusion, the inflammatory cytokine TNF contributes to elevated systemic FGF23 levels and triggers also ectopic renal *Fgf23* expression in CKD animal models.



# Keywords

Fibroblast growth factor 23 (FGF23), tumor necrosis factor (TNF), chronic kidney disease (CKD), inflammation, cytokine, inflammatory bowel disease, bone.

# INTRODUCTION

Chronic kidney disease (CKD) causes a severe disturbance of mineral metabolism, one of the leading factors for morbidity and mortality in patients with end stage renal disease (ESRD) [1, 2]. Fibroblast growth factor 23 (FGF23) increases early during CKD progression and is required to maintain serum phosphate levels while kidney function declines . In CKD patients, high FGF23 levels are associated with an increased risk of mortality independent of plasma phosphate [3]. FGF23 promotes left ventricular hypertrophy in rodents [4] and elevated FGF23 is a risk factor in the general population for all-cause and cardiovascular mortality [5].

FGF23 is critical for the regulation of phosphate homeostasis and vitamin $D_3$ metabolism . The main target organ of FGF23 is the kidney where FGF23 binds together with αKlotho to FGF receptors and inhibits phosphate reabsorption and decreases 1,25-$(OH)_2$ vitamin $D_3$ (1,25$(OH)_2$D) [6, 7]. FGF23 levels are regulated by a variety of stimuli including calcitriol, PTH, insulin, aldosterone, erythropoietin, and adipokinines [6, 8-11]. Moreover, FGF23 may be linked to inflammation. In the *Chronic Renal Insufficiency Cohort* elevated FGF23 is independently associated with higher IL-6 and TNF and also in a smaller cohort with only 103 CKD patients, RANTES and IL-12 associated with higher FGF23 [12]. The association between FGF23 and inflammation markers is not limited to CKD. *The Reasons for Geographic and Racial Differences in Stroke* study found a positive correlation of FGF23 with IL-6 and IL-10 in a non-CKD population [13]. Children during an acute phase of inflammatory bowel disease (IBD) had elevated FGF23 that normalized in the remission phase [14]. Furthermore, chondrocytes from patients with osteoarthritis have elevated *Fgf23* gene expression [15]. Microarray data from mouse models with FGF23 excess (*Col4a3* KO, *Hyp*, and *Fgf23* transgenic mice) show an activation of genes important in the regulation of the inflammatory response such as transforming growth factor beta (TGFβ), tumor necrosis factor (TNF) and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) [16]. Further, inflammatory stimuli and the hypoxia inducible transcription factor HIF-1 enhance FGF23 expression: TNF and TGFβ2

increases FGF23 expression in bone cells *in vitro* and HIF-1, interleukin-1 beta (IL-1β), lipopolysaccharide (LPS) increase FGF23 expression *in vitro* and *in vivo* [17-22]. Also, in an obesity induced model, TNF is necessary for the increase in FGF23 levels . Some inflammatory stimuli, including TNF, may act on *Fgf23* transcription via a 16 kb enhancer element . Moreover, in the folic-acid induced AKI model as well as in the adenine CKD model, genetic ablation of Il-6 reduced the increase in FGF23 . Thus, inflammatory cytokines may play an important role at least in the early phase of CKD to induce FGF23. However, whether TNF is a critical player has not been demonstrated.

Here, we investigated the association between inflammatory cytokines with plasma FGF23 in a population-based cohort and evaluated the effect of TNF on the regulation of plasma FGF23 in CKD animal models and in a non-renal inflammation model. Furthermore, we evaluated the role of hypoxia on *Fgf23* gene expression. Our results demonstrate a critical role for TNF to stimulate FGF23 in models of renal and non-renal inflammatory diseases.

# Results

## Plasma TNF positively correlated with intact FGF23 in the SKIPOGH population based cohort

The *Swiss Kidney Project on Genes in Hypertension* (SKIPOGH) is a family and population-based, multicenter, cross-sectional study including 1131 subjects randomly selected [23]. We assessed the relationship between plasma intact FGF23 (iFGF23) and parameters of phosphate metabolism, inflammatory cytokines, and iron metabolism while considering familial correlation. Participants with drugs interacting with calcium, magnesium and phosphate metabolism, inflammation and iron metabolism or have diuretic action were excluded. Based on a linear mixed model with family as random effect, $1,25(OH)_2D$, 25-(OH) vitamin $D_3$ (25(OH)D), TNF and calcium showed the highest fixed effects and were considered significant predictors of plasma iFGF23 while holding all the other variables constant (Figure 1). The standard deviation of the random effect was low compared to the standard deviation of the residuals (0.26 vs 0.93), which means that most of the variation in iFGF23 levels was due to the fixed effects (i.e. hormones, cytokines, etc.). There was no correlation between plasma iFGF23 and plasma phosphate, PTH, or eGFR. Besides TNF, no other inflammatory cytokine such as interferon gamma (IFNγ), IL-1β, IL-6, or IL-10 correlated with plasma iFGF23.

We also analyzed the cohort without applying exclusion criteria based on drugs. $1,25(OH)_2D$, 25(OH)D, and calcium remained as predictors of iFGF23 while phosphate, PTH and eGFR arose as additional predictors of iFGF23 (Figure S1). The TNF effect on iFGF23 is reduced in this population. . First quartile, median, mean and third quartile of continuous

variables in the SKIPOGH population with and without drug intake criteria applied are listed in Tables S1 and S2.

## Inflammation in kidneys of *Pkd1* conditional KO mice

TNF is increased in CKD patients, stimulates FGF23 expression in an osteocyte cell line, and was the only inflammatory cytokine associated with iFGF23 in the SKIPOGH cohort [19, 24-26]. Thus, we tested in two CKD mouse models whether TNF contributes to the rise of iFGF23 during the early phase of kidney disease. First, slowly progressing polycystic kidney disease (PKD) was induced in *Pkd1* conditional KO mice . Kidney function and two-kidney per body weight ratio were similar in 6 week old mice whereas kidney function was decreased and two-kidney per body weight ratio was increased in 12 week old *Pkd1*, *cre*+ mice (Figure S2). At week 6, iFGF23, TmP/GFR as well as renal *Tnf* and *Tgfb* mRNA expression were similar in $Pkd1^{fl/fl}$, *cre*- and $Pkd1^{fl/fl}$, *cre*+ mice (Figure 2 a - d). Progression of kidney disease was accompanied by increased plasma iFGF23, decreased TmP/GFR as well as increased *Tnf* and *Tgfb* mRNA expression in $Pkd1^{fl/fl}$, *cre*+ mice (Figure 2 a - d). TNF binding to TNF receptors activates the NFκB signaling pathway. The ratio of phospho-NFκB p65 to total NFκB p65 protein in the nuclear fraction of total kidney was significantly elevated in $Pkd1^{fl/fl}$, *cre*+ mice (Figure 2 e). Increased renal inflammatory cytokines in 12 week old $Pkd1^{fl/fl}$, *cre*+ mice were paralleled by the appearance of renal *Fgf23* expression and by the upregulation of the osteogenic marker gene *Runx2* in the kidney (Figure 2 f and S2 e). Bone *Fgf23* and *Runx2* mRNA expression were unchanged (Figure 2 g and S2 f).

## TNF blockade in *Pkd1* conditional KO mice suppressed FGF23

The effect of acute TNF blockade on FGF23 expression in PKD kidneys and on plasma iFGF23 was investigated. We injected intraperitoneally (i.p.) a single dose of 0.5 mg anti-TNF antibody or isotypic IgG control into 12 week old $Pkd1^{fl/fl}$, *cre*+ and $Pkd1^{fl/fl}$, *cre*- mice. After 24 hours, anti-TNF treated mice had a significant reduction of plasma TNF compared to the IgG control treated mice confirming the efficacy of the anti-TNF antibody (Figure 3 a). There was no difference in plasma TNF between IgG control treated $Pkd1^{fl/fl}$, *cre*+ and $Pkd1^{fl/fl}$, *cre*- mice. Importantly, elevated plasma iFGF23 in $Pkd1^{fl/fl}$, *cre*+ mice

was normalized by anti-TNF but not IgG control treatment (Figure 3 b). Plasma C-terminal FGF23 (cFGF23) was increased in IgG control treated *Pkd1*$^{fl/fl}$*, cre*+ and anti-TNF treated *Pkd1*$^{fl/fl}$*, cre*- compared to IgG control treated *Pkd1*$^{fl/fl}$*, cre*- mice consequently the iFGF23/cFGF23 ratio was elevated in IgG control treated *Pkd1*$^{fl/fl}$*, cre*- mice (Figure S4 a – c). There was no change in plasma phosphate and urea.(Figure 3 c and d). The abundance of the sodium dependent phosphate co-transporter NaPi-IIa in the brush border membrane (BBM) showed a trend to increase in *Pkd1*$^{fl/fl}$*, cre*+ mice when treated with anti-TNF antibodies (Figure 3 e). In *Pkd1*$^{fl/fl}$*, cre*+ mice, TNF neutralization decreased ectopic renal *Fgf23* mRNA expression while *Fgf23* mRNA expression in bone (Figure 3 f and g) and *Tnf*, and *Tgfb* mRNA expression in kidney (Figure 3 h and i) were unchanged. The mRNA expression of the inflammatory cytokines *Il1b* and *Il6* was elevated in PKD kidneys but did not change with anti-TNF treatment (Figure S3).

The orphan nuclear receptor Nurr1 is downstream of TNF signaling and activates *Fgf23* mRNA expression in rat osteosarcoma cells upon PTH treatment [27, 28]. *Nurr1* mRNA was detected in mouse kidney and bone (Figure S5). In the kidney of 12 week old *Pkd1*$^{fl/fl}$*, cre*+ mice, *Nurr1* mRNA expression was upregulated and Nurr1 protein was predominantly localized in the cell nucleus compared to *Pkd1*$^{fl/fl}$*, cre*- mice where Nurr1 was mainly distributed in the cytoplasm (Figure S6). Further, nuclear Nurr1 staining in *Pkd1*$^{fl/fl}$*, cre*+ mice was often co-localized with FGF23.

## TNF but not hypoxia increased FGF23 levels

We evaluated the effect of systemic TNF administration on plasma iFGF23. Therefore we injected wild type mice for two consecutive days with 2 µg recombinant mouse TNF. After 48-hours, plasma iFGF23 increased while cFGF23 and the iFGF23/cFGF23 ratio were unchanged (Figure 4a and S4 d – f). Furthermore plasma TNF and fractional excretion of phosphate increased, plasma urea decreased while plasma phosphate and creatinine levels were unchanged (Figure 4 b - f). In bone and spleen *Fgf23* mRNA expression decreased in TNF injected compared to vehicle injected mice whereas *Fgf23*

mRNA expression in thymus and bone marrow was unchanged (Figure 4 g -j). We cultured primary osteocytes from tibias and femurs of mice [29, 30] for 2 weeks before being supplemented for 24 hours either with 10 ng/ml TNF or 10 nM 1,25$(OH)_2$D. TNF as well as 1,25$(OH)_2$D increased *Fgf23* mRNA expression (Figure 4 k). TNF and 1,25$(OH)_2$D decreased the expression of *Dmp1* (Figure 4 l). Dmp1 inhibits *Fgf23* gene expression and loss of *DMP1* in patients causes hypophosphatemic rickets due to high FGF23 levels . TNF but not 1,25$(OH)_2$D increased the expression of *Galnt3* and *Nurr1* (Figure 4 m and n). Galnt3 mediates O-glycosylation of FGF23 preventing proteolytic cleavage of FGF23 **Error! Reference source not found.Error! Reference source not found.**.

CKD kidneys are commonly affected by hypoxia [31, 32] which was recently suggested to stimulate FGF23 expression through the hypoxia inducible transcription factor HIF-1 [17, 18, 21]. We studied in MC3T3-E1 mouse preosteoblasts the effect of hypoxia on *Fgf23* gene expression. MC3T3-E1 did not display intrinsic *Fgf23* expression. Nevertheless, after 2 weeks osteogenic differentiation of MC3T3-E1, *Fgf23* mRNA expression was induced by 10 nM 1,25$(OH)_2$D. 1,25$(OH)_2$D-induced *Fgf23* mRNA expression was completely repressed by hypoxic conditions (0.2% $O_2$) for 24 or 48 hours and hypoxia alone failed to trigger *Fgf23* expression (Figure S7 a). The upregulation of the HIF-1 target genes carbonic anhydrase 9 (*Car9*) and prolyl hydroxylase domain containing protein 2 (*Phd2*) confirmed the presence of hypoxia (Figure S7 b and c). Similarly, hypoxia had no effect on *Fgf23* mRNA expression in U2OS rat osteosarcoma and primary osteoblast cells (data not shown). We analyzed also kidneys of von Hippel-Lindau (*Vhl*) KO animals [33]. Lack of VHL prevents HIF hydroxylation and degradation and activates hypoxia sensitive genes [34]. Neither the kidneys of *Vhl* KO animals nor primary kidney cells lacking *Vhl* [35] expressed any detectable *Fgf23* (data not shown).

## TNF blockade lowers FGF23 levels in mouse models of oxalate nephropathy and colitis

We expanded our observations to another CKD mouse model, the oxalate nephropathy model in order to test for the relationship between TNF and FGF23 in a non-genetically modified mouse model and during early stages of kidney disease [36]. After induction of oxalate nephropathy, 48 hours prior to sacrifice, mice received a single i.p. injection of 0.5 mg anti-TNF or isotypic IgG control antibodies. IgG injected oxalate nephropathy mice had elevated plasma iFGF23 compared to control mice and TNF blockade normalized the elevated plasma iFGF23 in oxalate nephropathy mice (Figure 5 a). Plasma cFGF23 and iFGF23/cFGF23 did not differ between the groups (Figure S4 g – i). Plasma TNF was significantly reduced in the anti-TNF treated groups confirming the efficacy of the anti-TNF antibody (Figure 5 b). There was no difference in plasma TNF between IgG control treated oxalate nephropathy and control mice. Renal *Tnf* mRNA expression showed a trend to increase in oxalate nephropathy mice and was not affected by the anti-TNF antibody (Figure 5 c). There was no change in plasma phosphate and urine phosphate per urine creatinine ratio while the renal function parameters plasma creatinine and urea showed a trend to increase in the oxalate nephropathy mice (Figure 5 d – g).

To demonstrate that TNF regulates plasma iFGF23 independent from impaired kidney function, we analyzed a non-renal inflammation model, the *Il10* KO mouse developing spontaneously colitis [37]. Twelve to fourteen weeks old *Il10* KO mice had elevated plasma iFGF23 and increased colon *Tnf* mRNA expression (Figure 6 a and b). After 48 hours of a single i.p. injection of 0.5 mg anti-TNF or IgG control, anti-TNF treated *Il10* KO mice had reduced plasma iFGF23 compared to IgG treated animals whereas cFGF23 levels were similar (Figure 6 c and S4 k). There was a reduction in the iFGF23/cFGF23 ratio in anti-TNF treated *Il10* KO compared to IgG control treated *Il10* KO mice (Figure S4 l). Anti-TNF treatment had no effect on plasma phosphate levels (Figure 6 d) or kidney function parameters (Figure 6 e and f). But there was an increase in abundance of NaPi-

IIa at the BBM in *Il10* KO mice treated with anti-TNF antibodies compared to IgG control mice (Figure 6 g)

# Discussion

We provide a novel explanation for high iFGF23 levels in patients with chronic kidney disease or inflammation of non-renal origin. Our data demonstrate that TNF is positively and independently associated with plasma iFGF23 in humans. We show that exogenous TNF stimulates iFGF23 expression both *in vivo* and *in vitro*. TNF neutralization suppresses plasma iFGF23 in two CKD mouse models and triggers renal *Fgf23* expression in PKD kidneys. TNF also contributes to high iFGF23 in a model of intestinal inflammation with normal kidney function.

In humans, TNF levels correlated with plasma iFGF23 in the SKIPOGH multi-centric population based cohort. Dhayat et al. found in the same cohort associations between cFGF23 and plasma phosphate, $1,25(OH)_2D$, 25(OH)D, the ratio of TmP/GFR, age, sex, and renal function. However, there are relevant differences between both analyses: 1) we have measured both the biologically active iFGF23 and the biologically inactive C-terminal fragment, while Dhayat et al. [38] used a method that detects the sum of the intact form and the C-terminal fragment. 2) in addition to the subjects excluded by Dhayat et al. we excluded individuals taking drugs interacting with inflammation and subjects without complete data available for all variables. However, both analyses identified $1,25(OH)_2D$ and 25(OH)D as strong predictors of FGF23 variation in the SKIPOGH population while the correlation of PTH and eGFR in our study was dependent on drug exclusion criteria. The overall effect of TNF on iFGF23 may explain only a small part of the overall variability of iFGF23 in this cohort.

TNF increases in kidney disease and associates with CKD progression [24-26]. TNF stimulates *Fgf23* mRNA expression in an osteocyte-derived cell line [19] and may be involved in obesity induced increases in FGF23 . We tested the relevance of FGF23 regulation by TNF in pathological situations such as kidney disease or colitis. We used two distinct CKD mouse models, the *Pkd1* conditional KO mouse and the oxalate nephropathy model. PKD kidneys are affected by inflammation [39, 40] as confirmed by

higher renal *Tnf* and *Tgfb* expression as well as enhanced NFκB subunit p65 phosphorylation. Similarly, in oxalate nephropathy the inflammasome is activated and various proinflammatory cytokines are released [36, 41]**Error! Reference source not found.**. Ectopic renal FGF23 gene and protein expression occurs in rodents with either diabetic nephropathy, PKD, or 5/6 nephrectomy [42-44]. The increase of renal *Tnf* and *Tgfb* mRNA expression in PKD kidneys was paralleled by the increase in plasma iFGF23 levels, and the appearance of renal *Fgf23* and *Runx-2* expression. Renal FGF23 production may promote inflammation and fibrosis in the affected kidney [45-47]. We did not detect any change in bone *Fgf23* mRNA expression or plasma TNF levels in both CKD models. Similarly, in *Col4a3* KO mice, another CKD model, the early rise in plasma FGF23 is not accompanied by increased *Fgf23* expression in bone [48]. TNF blockade in both CKD models normalized plasma iFGF23 levels without changes in plasma phosphate levels. In the PKD model, TNF neutralization also reduced renal *Fgf23* expression. TNF may regulate renal *Fgf23* expression through NFκB stimulating orphan nuclear receptor *Nurr1* gene expression [28]. Nurr1 mediates the PTH dependent regulation of *Fgf23* in bone [27]. *Nurr1* was upregulated in PKD kidneys and predominantly localized in the cell nucleus whereas in wild type kidneys it was localized in the cytoplasm. Nurr1 nuclear localization often overlapped with renal FGF23 protein expression. Thus, Nurr1 may contribute to renal FGF23 expression.

In patients with CKD, TNF increases with ascending FGF23 quartiles and correlates with FGF23 levels independent of renal function and measures of mineral metabolism . Likewise, markers of inflammation correlate with ascending FGF23 quartiles in non-CKD stroke patients [13]. Inoculation of mice with LPS or bacteria stimulates serum FGF23 levels [20, 49]. In the diabetic nephropathy rat model, renal FGF23 was reduced by ramipril, an angiotensin-converting enzyme inhibitor , which also reduces inflammation [50]. Non-renal diseases characterized by inflammation such as inflammatory bowel disease (IBD) or osteoarthritis are linked to elevated plasma FGF23 [14, 15]. Patients with IBD or mouse colitis models show elevated FGF23 levels, lower 1,25$(OH)_2$D and impaired intestinal

phosphate absorption [14, 51-54]**Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.**. These disturbances are partially caused by TNF and in patients with IBD, TNF neutralizing therapy can reverse some of these abnormalities. We tested whether inflammation *per se* without renal disease could increase FGF23. Consistently, in *Il-10* KO mice, a model of IBD, plasma FGF23 increased and was reduced by TNF neutralization without affecting renal function parameters. Thus, extrarenal inflammation also stimulates FGF23 levels in mouse models and may play a role in humans.

David et al. reported that 6 hours after administration of the inflammatory cytokine IL-1β only cFGF23 increased while it required 4 days of consecutive IL-1β injections to increase also iFGF23 levels [21], whereas Onal et al showed higher FGF23 levels already 3 hours after IL-1β injection [49]. We demonstrate that TNF administration in wild type mice stimulated plasma iFGF23 levels within 48 hours without altering plasma phosphate and creatinine but increasing fractional excretion of phosphate demonstrating that iFGF23 is functional. TNF may exert even faster effects as indicated by higher FGF23 levels in mice 3 hours after TNF injection . The stimulation of *Fgf23* mRNA expression by TNF was confirmed *in vitro* in primary mouse osteocytes and comparable to the effect of $1,25(OH)_2D$. TNF but not $1,25(OH)_2D$ increased *Nurr1* and *Galnt3* expression in primary osteocytes suggesting that TNF but not $1,25(OH)_2D$ may regulate *Fgf23* expression in a Nurr1-dependent manner. TNF may also modulate FGF23 protein stability by regulating the expression of *Galnt3* which mediates the O-glycosylation of FGF23 making it more resistant to proteolytic degradation . In bone, C-terminal DMP-1 binds to PHEX and thereby inhibits *Fgf23* expression [55]. In primary osteocytes, *Dmp1* expression was strongly decreased by TNF and $1,25(OH)_2D$. The upregulation of *Fgf23* expression by TNF and $1,25(OH)_2D$ is paralleled by the downregulation of its suppressor. Our data expand previous observations in IDG-SW3 mouse osteocyte cells where TNF, IL-1β, and LPS increased *Fgf23* and reduced *Dmp1* mRNA expression [19]. TNF also stimulated *Fgf23* mRNA expression in rat UMR106 osteosarcoma cells and is required to increase

circulating FGF23 levels in a mouse obesity model . Deletion of an 16kb enhancer element in the *Fgf23* murine gene abolishes TNF induced FGF23 increases and reduces the effect of LPS and IL-1β on circulating FGF23 levels without altering bone structure or plasma phosphate and PTH. Induction of *Fgf23* mRNA in various organs is organ-specifically responsive to LPS, TNF and IL-1β and the deletion of the enhancer suggesting a complex and cell- and/or organ-specific regulation [49]. The enhancer element is also required for the early induction of FGF23 in the oxalate nephropathy model [49]. Thus, our work demonstrates the critical role of TNF in inducing FGF23 production and thereby complements previous work that identified a genetic element responding to TNF and possibly other regulators of *Fgf23* mRNA transcription . Furthermore, we expand these observations from kidney disease to at least one other clinically important condition, inflammatory bowel disease.

IL-6 has been recently identified as another important proinflammatory cytokine that associates with FGF23 levels in the CRIC cohort and that stimulates *Fgf23* mRNA in the IDG-SW3 osteocyte cell line [19]. Durlacher-Betzer et al. showed increased expression of IL-6 in kidney of folic-acid and adenine AKI and CKD mouse models and a partly blunted increase of circulating FGF23 levels in Il-6 deficient mice treated with adenine . While IL-6 may participate in the regulation of FGF23 in CKD, IL-6 plays also an important role in normal bone biology and IL-6 deficient mice have altered bone architecture [56, 57]. Thus, IL-6 may contribute to the upregulation of FGF23 in early CKD but TNF may act either upstream or is a critical permissive factor as indicated by the complete normalization of FGF23 levels in our experiments. In our population-based cohort, TNF but not IL-6 associated with intact FGF23 levels further strengthening the concept that TNF may play a central role in mediating effects of inflammation on bone.

Renal hypoxia is a common complication in CKD kidneys [31, 32]. Hypoxia increased *Fgf23* expression in UMR-106 rat osteosarcoma cells, and plasma cFGF23 but not iFGF23 in rats under hypobaric hypoxia conditions [18]. We cultured MC3T3-E1 cells, a mouse

preosteoblast cell line and primary mouse osteoblasts for 24 and 48 hours in 0.2% hypoxia and we did not observe any stimulation of *Fgf23* expression. In contrast, hypoxia suppressed the stimulatory effect of $1,25(OH)_2D$ on *Fgf23*. TNF and IL-1β increase HIF-1 binding to DNA under normoxia while in combination with hypoxia both cytokines strongly increase HIF-1 activity [58]. IL-1β but not TNF enhance nuclear accumulation of HIF-1α in a hepatoma cell line [58] and increase FGF23 mRNA expression in bones and kidneys [21]. Inhibition of HIF-1α attenuated the positive effect of IL-1β on FGF23 expression [21]. Combined with the fact that we did not find any effect of constitutively activated HIF-1α in *Vhl* KO animals as well as in primary kidney cells lacking *Vhl*, these results suggest that the HIF-1α mediated upregulation of *Fgf23* expression may depend on IL-1β or other factors such as erythropoietin [9, 21, 59, 60].

In summary, TNF stimulates iFGF23 in renal and non-renal inflammatory mouse models and in primary bone cell culture; triggers renal *Fgf23* expression in CKD animal models and is positively associated with plasma iFGF23 in a population-based cohort. These findings question the concept that the early rise in plasma FGF23 in CKD is solely to balance plasma phosphate while kidney function declines. The data suggest that other non-renal inflammatory processes may strongly impact on plasma FGF23 levels. Our study suggests novel therapeutic options to reduce excessive FGF23 levels in kidney and other diseases as drugs lowering TNF are widely clinically used and have proven to be safe in humans.

# Methods

## SKIPOGH cohort

We obtained 1098 out of 1131 human EDTA plasma samples from SKIPOGH cohort (Swiss Kidney Project on Genes in Hypertension) [23, 61, 62]. Plasma iFGF23 was measured with the human intact FGF23 ELISA kit (Immutopics International, USA). For statistical modeling the following 18 previously determined parameters were used: plasma calcium, phosphate, ferritin, transferrin, iron, $1,25(OH)_2D$, 25(OH) vitamin $D_3$, PTH, TNF, IFNγ, IL-1β, IL-6, IL-10 and cFGF23 as well as body mass index, age, sex and estimated renal function calculated by the CKD-EPI equation.

Exclusion criteria followed the pipeline described in the Table S3. Participants with incomplete data sets (n = 261) were excluded. TNF followed a bimodal distribution with 40 values close to undetectable (TNF < 1 pg/ml) without continuity with the rest of the distribution, highly suggestive for measurement failures. Therefore the 40 participants with TNF < 1 pg/ml were excluded from the study. Next, the ratio between iFGF23 (detects only iFGF23) and cFGF23 (detects iFGF23 and cFGF23) was calculated. One Ru/ml cFGF23 corresponds to 1.5 pg/ml iFGF23 (information provided by Immutopics); participants with ratios higher than 1.5 were excluded (n = 40). To avoid confounding effects by drug intake we eliminated 4 major drug categories that interact with FGF23 metabolism: 1) calcium, phosphate and magnesium (n = 41); 2) inflammation (pro or anti-inflammatory) (n = 390); 3) iron metabolism (n = 6); 4) kidney function (i.e. diuretics) (n = 54) (Table S4). A total of 361 participants were excluded due to intake of drugs of one or more of these drug categories. The final dataset contains either 429 participants (198 female / 231 male) with or 790 (424 female / 366 male) without drug exclusion criteria.

## Animals

*Pkd1* floxed/floxed (*Pkd1*$^{fl/fl}$) tamoxifen inducible *cre* mice were kindly provided by Gregory Germino [63, 64]. *Cre* recombinase expression is under the control of the β-actin promoter which drives high levels of expression in most tissues. Male and female *Pkd1*$^{fl/fl}$,

*cre+* and *Pkd1*$^{fl/fl}$*, cre-* mice were used. Cre recombinase activity was induced at postnatal days 15, 17, and 19 by injecting pups with 100 µl tamoxifen (2.5 mg/ml) in corn oil causing slow onset disease. Without further interventions, 24-hour urine was collected from 6 and 12 weeks old animals (e.g. 3 or 9 weeks after induction, respectively) which were thereafter sacrificed to collect plasma and organs. For TNF blockade, animals were treated at the age of 11-12 weeks with a single i.p. injection of 0.5 mg *InVivo*MAb anti-Tnfα (Clone XT3.11, Lot4653-1/0413, BioXCell, USA) or *InVivo*MAb rat IgG1 (Clone HRPN, Lot 5339/1014, BioXCell, USA) [65, 66]. Twenty-four hours after antibody application, animals were sacrificed and plasma and organs were collected. The effect of TNF in wild-type mice was assessed by injecting 13 weeks old C57Bl/6J mice on two consecutive days with 2 µg TNF. After 48 hours, plasma and organs were collected.

Nephropathy was induced in 10 to 12 weeks old C57Bl/6J mice. After 3 days of adaptation with calcium-free diet (irradiated S7042-E005S, Sniff Spezialdiäten GmbH, Germany), mice were fed for 10 days with either calcium free diet or 0.67% oxalate in calcium-free diet (irradiated S7042-E010) followed by a 5-day recovery phase in standard diet (3433, Kliba, Kaiseraugst, Switzerland). Forty-eight hours prior to sacrifice, mice received a single i.p. injection of 0.5 mg anti-TNF or isotypic IgG1 control. Mice were sacrificed and plasma and organs were collected.

*Il10* deficient mice (*Il10*$^{-/-}$) develop spontaneous colitis and were used as a non-renal inflammatory disease model [37]. *Il10*$^{-/-}$ mice between 12-14 weeks were sacrificed to collect plasma and organs. *Il10*$^{-/-}$ mice were treated with a single i.p. injection of 0.5 mg *InVivo*MAb anti-Tnfα (Clone XT3.11, Lot4653-1/0413, BioXCell, USA) or *InVivo*MAb rat IgG1 (Clone HRPN, Lot 5339/1014, BioXCell, USA) [65, 66] 48 hours prior to sacrifice and plasma and organs were collected. For some experiments, kidneys from kidney-specific von-Hippel-Lindau deficient mice were used [33]. All animal studies were performed according to protocols approved by the legal authority (Veterinary Office of the Canton of Zurich or the Committee on Animal Research, University of California San Francisco).

## Plasma and urine analysis

Blood and 24 hours urine were collected from *Pkd1*$^{fl/fl}$*, cre+* and *Pkd1*$^{fl/fl}$*, cre-* mice at 6 and 12 weeks after birth. Briefly, *Pkd1*$^{fl/fl}$*, cre* mice were kept for three days in metabolic cages (Tecniplast, Italy) whereas the last day was used for 24 hours urine collection. Afterwards mice were anesthetized with isoflurane and blood was collected from the heart. Plasma and urine aliquots were rapidly frozen and stored at -80 °C until measurement. Urine and plasma laboratory analyses were performed on a UniCel DxC 800 Synchron (Beckman Coulter, Switzerland) by the Zurich Integrative Rodent Physiology (ZIRP) core facility. The ratio of the maximum rate of tubular phosphate reabsorption to the glomerular filtration rate (TmP/GFR) was calculated as follows: $TmP/GFR\ in\ mmol/L = P_P - [U_P \times P_{crea}/U_{crea}]$[67]. The fractional excretion of phosphate was calculated according to the following equation: $FE_{Pi} = (U_{Pi} \times P_{crea}) = (P_{Pi} \times U_{crea}) \times 100$. $P_{Pi}$, $U_{Pi}$, $P_{crea}$, and $U_{crea}$ refer to the plasma and urinary concentration of phosphate and creatinine, respectively. The plasma concentration of intact FGF23 (Kainos Laboratories, Japan or Immutopics International, USA), cFGF23 (Immutopics International, USA), intact PTH (Immutopics International, USA) and TNF (Bio-Techne AG, Switzerland) were measured by enzyme-linked immunosorbent assays according to the manufacturers protocols.

## Cell culture

All cell culture reagents were from Life Technologies Europe B.V. (Switzerland) unless stated otherwise. Two to four month old *Pkd1*$^{fl/fl}$*, cre* mice (4-6 mice per experiment, male and female mixed) were sacrificed with carbon dioxide. Tibias and femurs from the hindlegs were harvested. The epiphyses were cut and bones were flushed with Hank's Balanced Salt Solution (HBSS) containing 1% penicillin streptomycin (Pen Strep) to remove the bone marrow. Bones were cut into small pieces of 1-2 $mm^2$. Bone cell extraction was performed according to established protocols [29, 30]. Briefly, small bone pieces were repeatedly digested with either a solution containing 2 mg/ml collagenase

type II, 0.05% (w/v) soybean trypsin inhibitor (Sigma-Aldrich, Switzerland), 20 mM HEPES, 1% Pen Strep in HBSS or 10 nM EDTA, 1% fetal bovine serum (FBS), 1% Pen Strep in phosphate buffered saline (PBS) for 25 min at 37°C. Cells from digestion steps 6-9 or cells and bone pieces from digestion step >9 were cultured for 2 weeks in an osteogenic medium (minimal essential medium α (memα) containing 10% FBS, 1% Pen Strep, 50 µg/ml 2-phospho-L-ascorbic acid trisodium salt (Sigma-Aldrich, Switzerland), and 1 mM β-glycerophosphate (Sigma-Aldrich, Switzerland)). After 2 weeks, cells were supplemented for 24 hours with either 10 nM 1,25$(OH)_2$D (CaymanChemical, USA) or 10 ng/ml mouse TNF (R&D Systems, USA) and total mRNA was extracted.

MC3T3-E1 subclone 4 preostoblast cells (CRL-2593, Lot 59899932, ATCC France) passage 17/4 were expanded for 4-5 days with MEMα medium supplemented with 10% FBS and 1% PenStrep. After reaching 80-90% confluence, MC3T3-E1 cells were trypsinized and plated in collagen coated 6-well plates (80'000 cells/well). Medium was changed to osteogenic differentiation medium (MEMα supplemented with 10% FBS, 1% PenStrep, 50µg/ml 2-phospho-L-ascorbic acid trisodium salt (Sigma-Aldrich, Switzerland), and 1 mM beta glycerophosphate (Sigma-Aldrich, Switzerland)). After 2 weeks differentiation along the osteogenic lineage cells were supplemented for 24 or 48 hours with either 10 nM 1,25$(OH)_2$D (CaymanChemical, USA) or an equal amount of ethanol and incubated for 24 or 48 hours under hypoxic (0.2% $O_2$) or normoxic conditions. Hypoxia experiments were performed in a gas-controlled workstation (Invivo$O_2$, Baker Ruskinn, UK).

## RNA extraction, reverse transcription and qPCR

Organs and scraped colonic mucosa were harvested and rapidly frozen in liquid nitrogen. Tissues were homogenized using either a Precellys homogenizer or a liquid nitrogen cooled mortar and pestle (bone). Total mRNA from bone as well as from cultured cells was extracted with TRIzol (Life Technologies Europe B.V., Switzerland) followed by purification with RNeasy Mini Kit (Qiagen, Switzerland) according to the manufacturers protocol. Total mRNA from kidney and colonic mucosa were extracted with RNeasy Mini

Kit (Qiagen, Switzerland) according to the manufacturer's protocol. DNAse digestion was performed using the RNase-free DNAase Set (Qiagen, Switzerland). Total RNA extractions were analyzed for purity and concentration using the NanoDrop ND-1000 spectrophotometer (Wilmington, Germany). RNA samples were diluted to a final concentration of 100 ng/µl and cDNA was prepared using the TaqMan Reverse Transcriptase Reagent Kit (Applied Biosystems, Roche, Foster City, CA). In brief, in a reaction volume of 40 µl, 300 ng of RNA was used as template and mixed with the following final concentrations of RT buffer (1x): $MgCl_2$ (5.5 mmol/l), random hexamers (2.5 µmol/l), dNTP mix (500 µmol/l each), RNase inhibitor (0.4 U/µl), multiscribe reverse transcriptase (1.25 U/µl), and RNAse-free water. Reverse transcription was performed with temperature conditions set at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min on a thermocycler (Biometra, Germany). Quantitative PCR (qPCR) was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers for genes of interest were designed using Primer 3 software. Primers were chosen to span exon - exon boundaries to exclude the amplification of contaminating genomic DNA (primer and probe sequence see Table S5). The specificity of all primers was tested and always resulted in a single product of the expected size (data not shown). Probes were labeled with the reporter dye FAM at the 5'-end and the quencher dye TAMRA at the 3'-end (Microsynth, Switzerland). qPCR reactions were performed using the KAPA PROBE FAST qPCR Kit (KappaBiosystems, USA) or PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Switzerland).

## Protein extraction and Western blot analysis

Organs were rapidly frozen in liquid nitrogen. Tissues were homogenized in homogenization buffer containing 0.27 M sucrose, 2 mM EDTA (pH8), 0.5% NP-40, 60 mM KCl, 15 mM NaCl, 15 mM HEPES (pH7.5) (all Sigma-Aldrich, Switzerland) and complete protease inhibitor cocktail (Roche, Switzerland) using Precellys homogenizer. Nuclei were separated by a sucrose cushion and resuspended in a nuclear extraction buffer containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA (pH 8), 1 mM DTT

and 1 mM PMSF (all Sigma-Aldrich, Switzerland). BBM vehicles were prepared using the $Mg^{2+}$ precipitation technique [68]. After measurement of protein concentration (Bio-Rad, Hercules, CA, USA), 60 µg of nuclear proteins or 20 ug of BBM proteins were solubilized in loading buffer containing DTT and separated on a 10% polyacrylamide gel. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline/0.1% Tween-20 or 5% bovine serum albumin (BSA) in Tris-buffered saline/0.1% Tween-20 for 60 min, blots were incubated with the primary antibodies: mouse monoclonal anti-phospho-NFκB p65 (Ser536)(7F1) (Cell Signaling Technology, USA; 1:1000), rabbit monoclonal NFκB p65 (D14E12) (Cell Signaling Technology, USA; 1:1000), rabbit polyclonal anti-NaPi-IIa ([69]; 1:3000) or mouse monoclonal anti-β-actin either for 2 h at room temperature or overnight at 4 °C. Membranes were then incubated for 1 h at room temperature with secondary goat anti-rabbit or donkey anti-mouse antibodies (1:5000) linked to alkaline phosphatase (Promega, USA) or HRP (Amersham, MA, USA or R&D Systems, USA). The protein signal was detected with the appropriate substrates using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analyzed using the software Advanced Image Data Analyser AIDA, Raytest to calculate the ratio between phosphorylated protein to total protein.

## Immunofluorescence staining

Mouse kidneys were perfused through the left heart ventricle with a fixative solution containing 3% paraformaldehyde in phosphate buffered saline (PBS). Kidneys were embedded in TissueTec and frozen in liquid nitrogen. Five µm cryosections were cut. Slides were rehydrated with PBS, treated for 5 min with 0.5% SDS in PBS followed by 10 min treatment with 0.5% Triton-X-100 in PBS (Sigma-Aldrich, Switzerland). Unspecific sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Primary antibodies were diluted in 1% BSA in PBS (rat anti-FGF23 clone #283507 (R&D Systems, USA) 1:1000; rabbit anti-Nurr1 N-20 sc-991 (Santa-Cruz, USA)

1:200) and kidney sections were incubated with the primary antibody overnight at 4 °C. After washing with PBS, sections were incubated with the corresponding secondary antibody (1:500) (anti-rabbit DyLight 594 (Jackson ImmunoResearch, Europe), anti-rat NL493 (R&D Systems, USA)), and DAPI (Life Technologies Europe B.V., Switzerland, 1:1000) for 1 h at room temperature. Slides were washed twice with PBS before they were mounted with Dako glycergel mounting medium (Dako, Switzerland). Sections were visualized on a Leica DM 5500B fluorescence microscope and images processed with ImageJ.

## Statistical analysis

Statistics were performed using unpaired Student's t-test, ANOVA, or Two-Way-ANOVA (GraphPad Prism version 7, GraphPad, San Diego, CA) and R programming environment including the nlme, visreg, data.table, car, lmtest, and forestplot packages**Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.**. $P < 0.05$ was considered significant.

The identification of predictors for iFGF23 variation in the SKIPOGH population was performed using linear mixed models with random intercept. The distribution of all parameters was analyzed in histograms. Due to a heavily skewed distribution, IL-6, IL-10, IFNγ and IL1-β were log-transformed. All parameters were centralized and then normalized by their standard deviations. Linear or nonlinear relationship of each variable with iFGF23 was assessed using a component residual plot. However, all parameters were considered linear. Assumptions on the within-group error were checked with plots of the standardized residuals versus fitted values and a Q-Q plot of the residuals. The assumptions on the random effects were checked with a Q-Q plot of the random effects.

## Author contributions

Conceptualization, D. E-S., P.H.I.S., and C.A.W; Methodology, D. E-S., P.H.I.S., and C.A.W; Formal analysis, D. E-S. and P.H.I.S.; Investigation, D. E-S., P.H.I.S., B.G., N.G.,

C.B., M.Z., D.S., M.R., D.A., B.P., M.P., A.L., V.B., and G.-M. F; Resources C.A.W., D.H., F.K., I.F-W., G.R., M. B., F.P., M.F., F.L., R.H.W., S.H.- and I.F.; Writing – Original Draft, D. E-S. Writing -Review & Editing, D. E-S., P.H.I.S., and C.A.W; Visualization, D. E-S. and P.H.I.S.; Supervision, C.A.W.; Funding Acquisition, C.A.W, all authors read, edited and approved the manuscript.

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University Hospitals, Geneva) and Menno Pruijm (Lausanne University Hospital, Lausanne).

## Conflict of interests

C.A. Wagner has been a member of an advisory board to Bayer Pharma AG, and provided consultancy to Medice. No other financial interests are reported.

## Figure legends

### Figure 1

**Identification of plasma iFGF23 predictors in a human cohort.** (a) Forest plot showing the fixed effects calculated for all predictors used in the mixed linear model for the subpopulation of 429 participants after all the exclusion criteria applied. Fixed effect estimates (β), standard error, ratio between the estimates and their standard errors (t-value), and associated p-value from a t-distribution. The parameters are ordered by fixed effect estimates. (b) Association between plasma TNF and iFGF23 in the SKIPOGH cohort in a subpopulation of 429 participants after all the exclusion criteria applied. The regression line and confidence band were obtained from the linear mixed model containing all the predictors.

### Figure 2

**FGF23 and inflammation in *Pkd1* KO mice.** Plasma FGF23 (a) and TmP/GFR (b) as well as renal *Tnf* (c) and renal *Tgfb* (d) mRNA expression relative to 18SrRNA in *Pkd1*$^{fl/fl}$, *cre*- (white squares) and *Pkd1*$^{fl/fl}$, *cre*+ (black squares) animals after 6 and 12 weeks. Phosphorylation of NFκB p65 (e) in the nuclear fraction of total kidney protein homogenates in *Pkd1*$^{fl/fl}$, *cre*- (white squares) and *Pkd1*$^{fl/fl}$, *cre*+ (black squares) animals after 12 weeks. Renal (f) and bone (g) *Fgf23* mRNA expression relative to 18SrRNA in *Pkd1*$^{fl/fl}$, *cre*- (white squares) and *Pkd1*$^{fl/fl}$, *cre*+ (black squares) animals after 12 weeks. ND = not detected. Two-way ANOVA with Bonferroni correction (a - d) or unpaired t-test (e - g), * $p<0.05$.

### Figure 3

**TNF neutralization lowers FGF23 in *Pkd1* KO mice.** Plasma TNF (a), iFGF23 (b), phosphate (c), and urea (d) levels, bone (e) and renal (f) *Fgf23*, renal *Tnf* (g), and renal *Tgfb* (h) mRNA expression relative to *Hprt* as well as abundance of NaPi-IIa (i) in the renal BBM relative to β-actin 24 hours after injection of 0.5mg isotypic IgG control or anti-TNF neutralizing antibodies in 11-12 weeks old *Pkd1*$^{fl/fl}$, *cre*- (white squares) and *Pkd1*$^{fl/fl}$, *cre*+ (black squares) animals. ND = not detected. Two-way ANOVA with Bonferroni correction * $p<0.05$.

### Figure 4

**TNF stimulates FGF23 *in vivo* and *in vitro*.** Plasma iFGF23 (a), TNF (b), phosphate (c), creatinine (d), urea (e) and $FE_{Pi}$ (f) as well as bone (g), spleen (h) thymus (i) and bone marrow (j) Fgf23 mRNA expression relative to *Hprt* (g,h) or *18SrRNA* (i,j) 48 hours after two consecutive injections of vehicle or 2 µg recombinant mouse TNF in 12 weeks old wild type mice. Unpaired t-test * $p<0.05$. Fold increase of *Fgf23* (k), *Dmp1* (l), *GalNt3* (m), and *Nurr1* (n) mRNA expression compared to untreated control in primary murine osteocytes after stimulation with $1,25(OH)_2D$ (white squares) or 10ng/ml TNF (black squares) for 24 hours. Single experiments were normalized to their untreated control (dashed line = 1). Number of independent experiments 9-10; One-way ANOVA with Bonferroni correction * $p<0.05$ compared to $1,25(OH)_2D$ treated cells, # $p<0.05$ compared to untreated cells.

**Figure 5**

**TNF neutralization lowers plasma iFGF23 in mice with oxalate nephropathy.** Oxalate-nephropathy was induced in wild type mice. Plasma iFGF23 (a), plasma TNF (b), renal *Tnf* (c) mRNA expression relative to *Hprt*, plasma phosphate (d), urinary phosphate to creatinine ratio (e), plasma creatinine (f) and plasma urea (g) 48 hours after injection of 0.5 mg isotypic IgG control or anti-TNF neutralizing antibodies in control diet (white squares) and oxalate nephropathy (black squares) induced mice. One-way ANOVA with Bonferroni correction * $p<0.05$.

**Figure 6**

**Colonic inflammation increases plasma iFGF23 via TNF in *Il-10* KO mice.** Plasma iFGF23 (a) levels and colonic *Tnf* (b) mRNA expression relative to 18SrRNA in 14 weeks old *Il-10*$^{+/+}$ and *Il-10*$^{-/-}$ mice. Plasma iFGF23 (c), phosphate (d), creatinine (e), and urea (f) levels as well as abundance of NaPi-IIa at the renal BBM 48 hours after injection of 0.5 mg isotypic IgG control or anti-TNF neutralizing antibodies in 12 weeks old *Il-10*$^{-/-}$ mice. Unpaired t-test * $p<0.05$.

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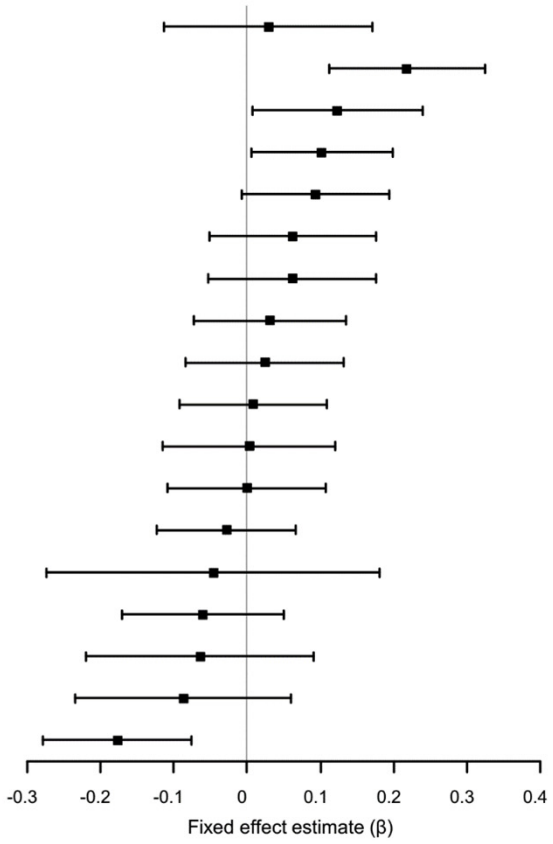
# Figure 1

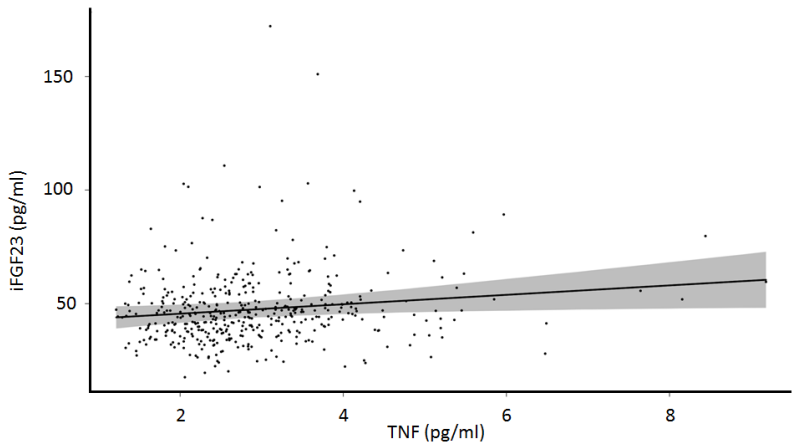
**a**

**With exclusion by drug intake**

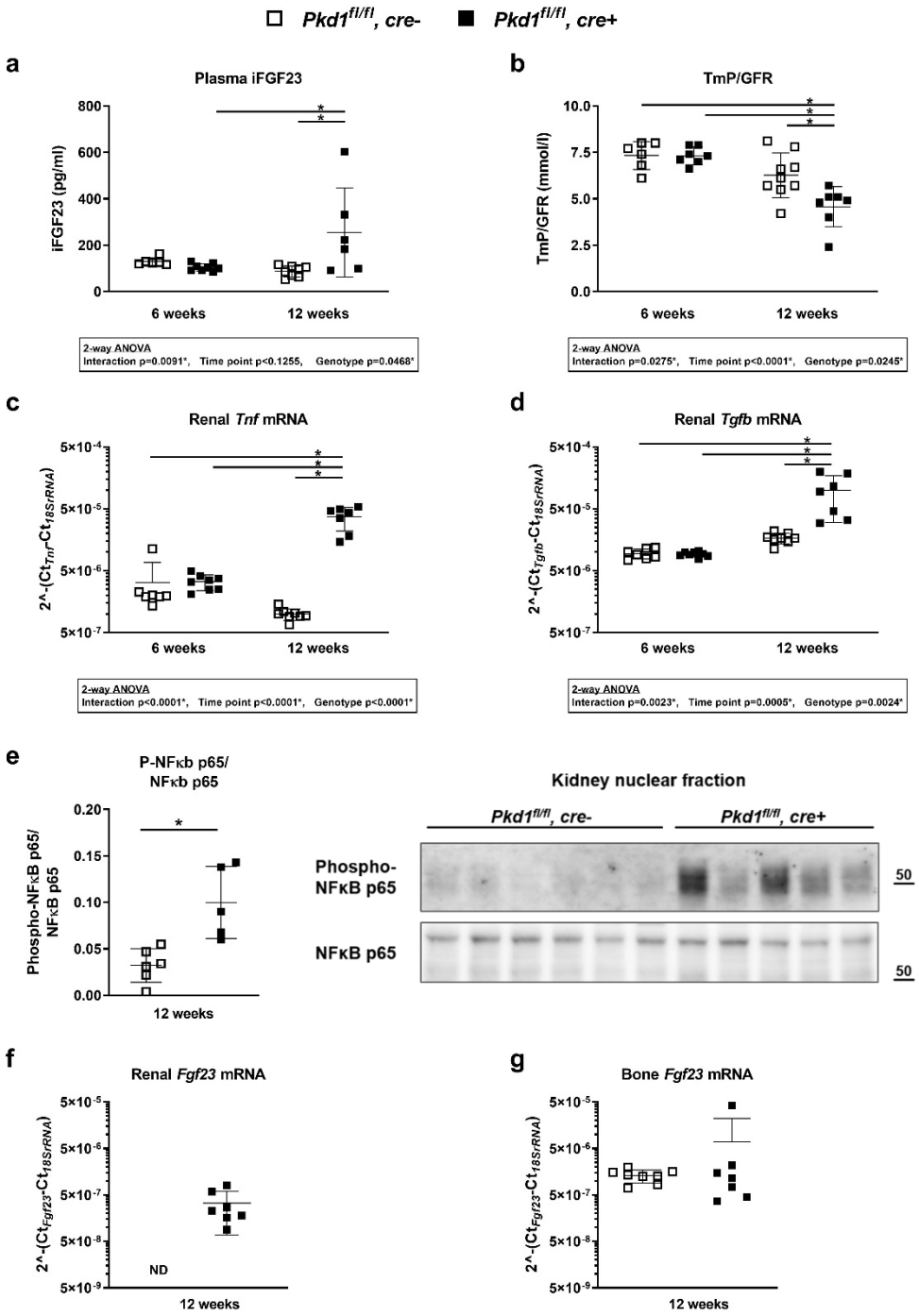
| | β | SE | t-value | p-value |
|---|---|---|---|---|
| Intercept | 0.029 | 0.072 | 0.394 | 0.694 |
| 25(OH)D | 0.218 | 0.054 | 4.047 | <0.001 |
| TNF | 0.123 | 0.059 | 2.110 | 0.036 |
| Calcium | 0.102 | 0.049 | 2.088 | 0.038 |
| Iron | 0.093 | 0.051 | 1.819 | 0.070 |
| IL-6 | 0.062 | 0.058 | 1.072 | 0.285 |
| PTH | 0.062 | 0.058 | 1.062 | 0.289 |
| Transferrin | 0.031 | 0.053 | 0.589 | 0.557 |
| BMI | 0.024 | 0.055 | 0.435 | 0.664 |
| Phosphate | 0.008 | 0.051 | 0.155 | 0.877 |
| IFNγ | 0.003 | 0.06 | 0.053 | 0.957 |
| IL-10 | 0 | 0.055 | -0.007 | 0.994 |
| IL-1b | -0.028 | 0.048 | -0.590 | 0.556 |
| Sex | -0.046 | 0.115 | -0.400 | 0.689 |
| Ferritin | -0.061 | 0.056 | -1.081 | 0.281 |
| Age | -0.064 | 0.078 | -0.812 | 0.418 |
| eGFR | -0.087 | 0.074 | -1.167 | 0.245 |
| 1,25(OH)2D | -0.177 | 0.051 | -3.460 | 0.001 |



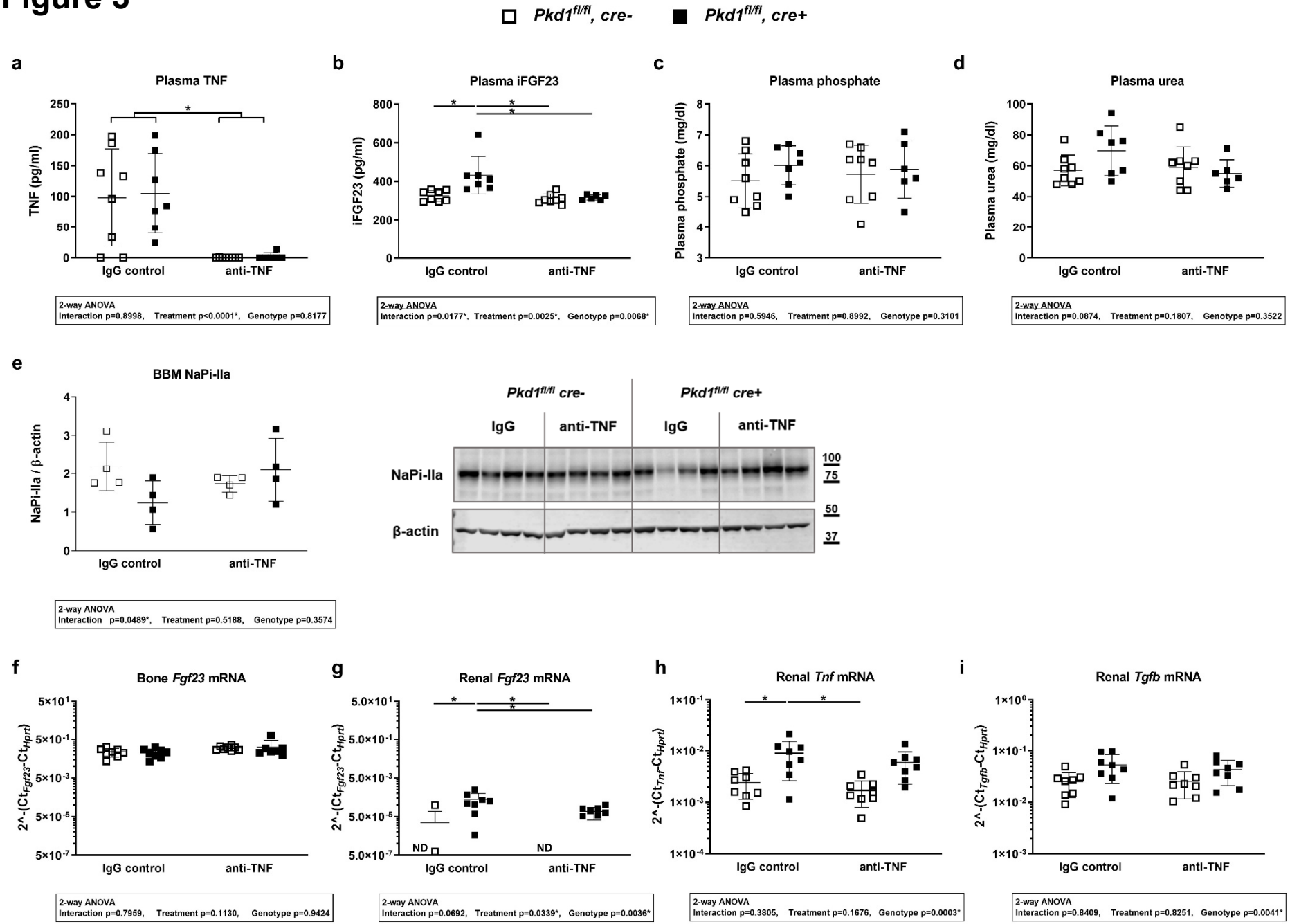

**b**



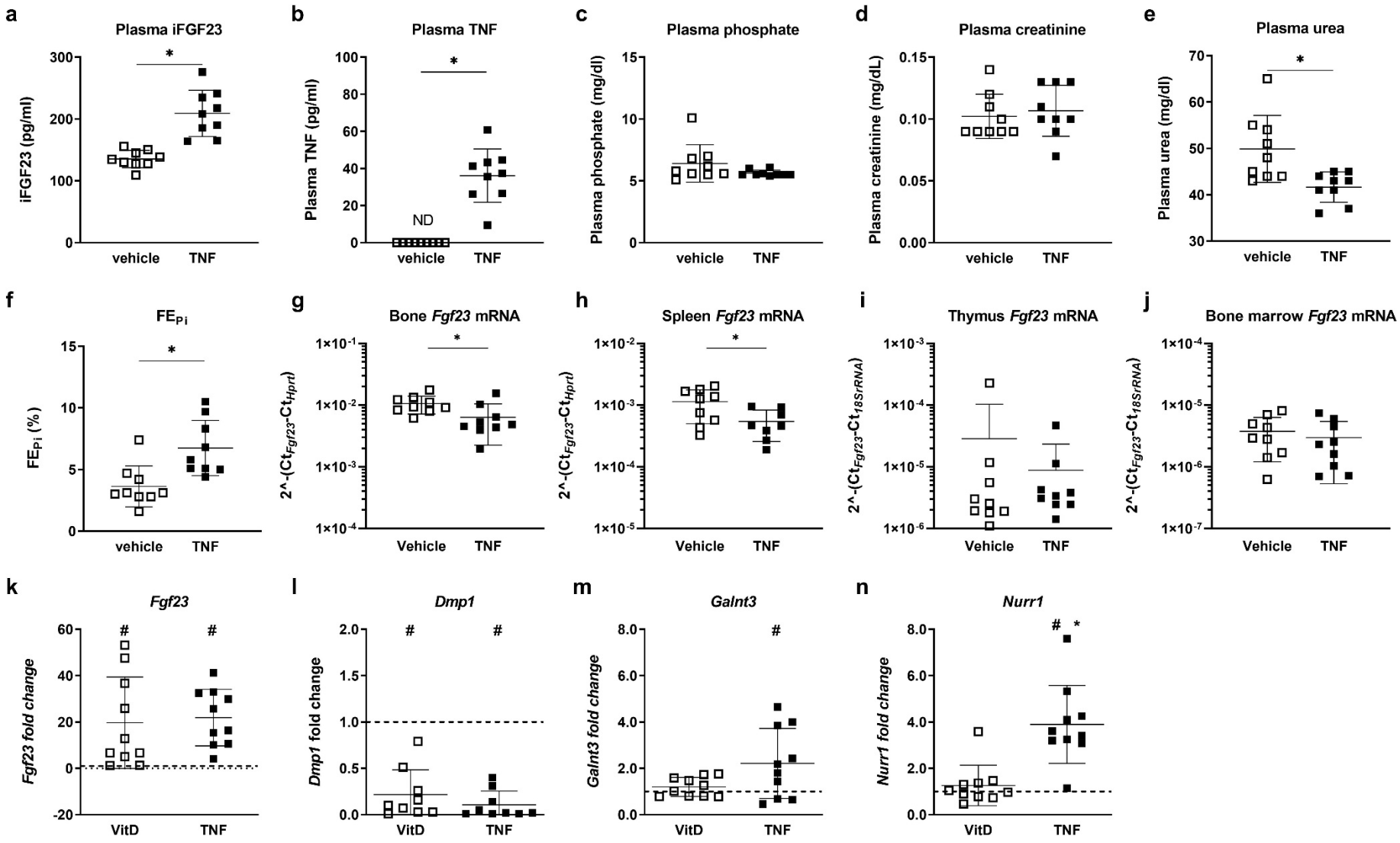
# Figure 2


Pkd1fl/fl, cre-
Pkd1fl/fl, cre+
a
Plasma iFGF23
iFGF23 (pg/ml)
800
600
400
200
0
6 weeks
12 weeks
2-way ANOVA
Interaction p=0.0091*, Time point p<0.1255, Genotype p=0.0468*
b
TmP/GFR
TmP/GFR (mmol/l)
10.0
7.5
5.0
2.5
0.0
6 weeks
12 weeks
2-way ANOVA
Interaction p=0.0275*, Time point p<0.0001*, Genotype p=0.0245*
c
Renal Tnf mRNA
2^-(Ct Tnf-Ct 18S rRNA)
5×10-4
5×10-5
5×10-6
5×10-7
6 weeks
12 weeks
2-way ANOVA
Interaction p<0.0001*, Time point p<0.0001*, Genotype p<0.0001*
d
Renal Tgfb mRNA
2^-(Ct Tgfb-Ct 18S rRNA)
5×10-4
5×10-5
5×10-6
5×10-7
6 weeks
12 weeks
2-way ANOVA
Interaction p=0.0023*, Time point p=0.0005*, Genotype p=0.0024*
e
P-NFκb p65/
NFκb p65
Phospho-NFκB p65/
NFκB p65
0.20
0.15
0.10
0.05
0.00
12 weeks
Kidney nuclear fraction
Pkd1fl/fl, cre-
Pkd1fl/fl, cre+
Phospho-
NFκB p65
NFκB p65
50
50
f
Renal Fgf23 mRNA
2^-(Ct Fgf23-Ct 18S rRNA)
5×10-5
5×10-6
5×10-7
5×10-8
5×10-9
ND
12 weeks
g
Bone Fgf23 mRNA
2^-(Ct Fgf23-Ct 18S rRNA)
5×10-5
5×10-6
5×10-7
5×10-8
5×10-9
12 weeks

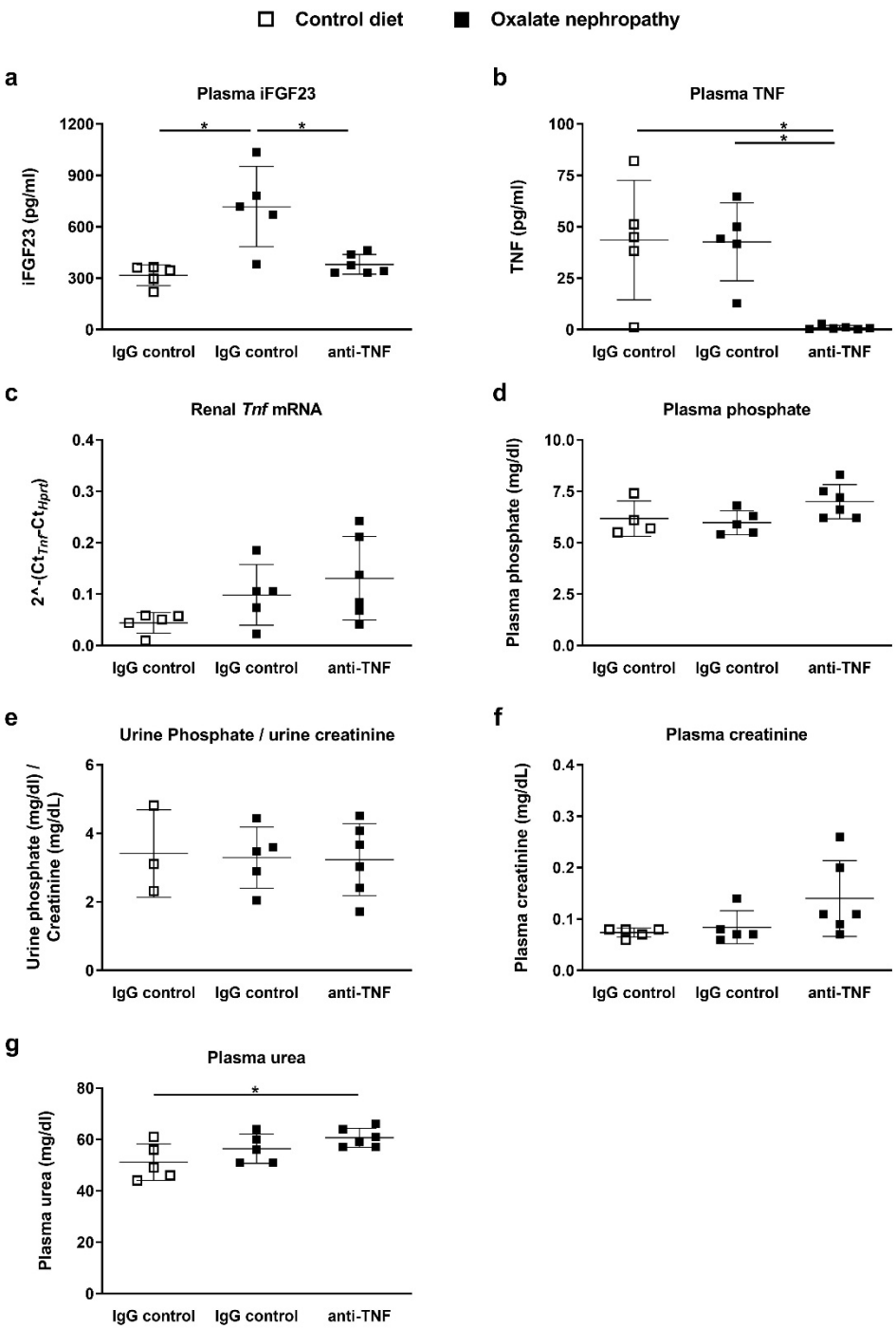
# Figure 3


Pkd1fl/fl, cre-
Pkd1fl/fl, cre+
a
Plasma TNF
TNF (pg/ml)
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.8998, Treatment p<0.0001*, Genotype p=0.8177
b
Plasma iFGF23
iFGF23 (pg/ml)
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.0177*, Treatment p=0.0025*, Genotype p=0.0068*
c
Plasma phosphate
Plasma phosphate (mg/dl)
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.5946, Treatment p=0.8992, Genotype p=0.3101
d
Plasma urea
Plasma urea (mg/dl)
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.0874, Treatment p=0.1807, Genotype p=0.3522
e
BBM NaPi-IIa
NaPi-IIa / β-actin
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.0489*, Treatment p=0.5188, Genotype p=0.3574
Pkd1fl/fl cre-
Pkd1fl/fl cre+
IgG
anti-TNF
IgG
anti-TNF
NaPi-IIa
β-actin
100
75
50
37
f
Bone Fgf23 mRNA
2^-(CtFgf23-CtHprt)
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.7959, Treatment p=0.1130, Genotype p=0.9424
g
Renal Fgf23 mRNA
2^-(CtFgf23-CtHprt)
ND
ND
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.0692, Treatment p=0.0339*, Genotype p=0.0036*
h
Renal Tnf mRNA
2^-(CtTnf-CtHprt)
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.3805, Treatment p=0.1676, Genotype p=0.0003*
i
Renal Tgfb mRNA
2^-(CtTgfb-CtHprt)
IgG control
anti-TNF
2-way ANOVA
Interaction p=0.8409, Treatment p=0.8251, Genotype p=0.0041*

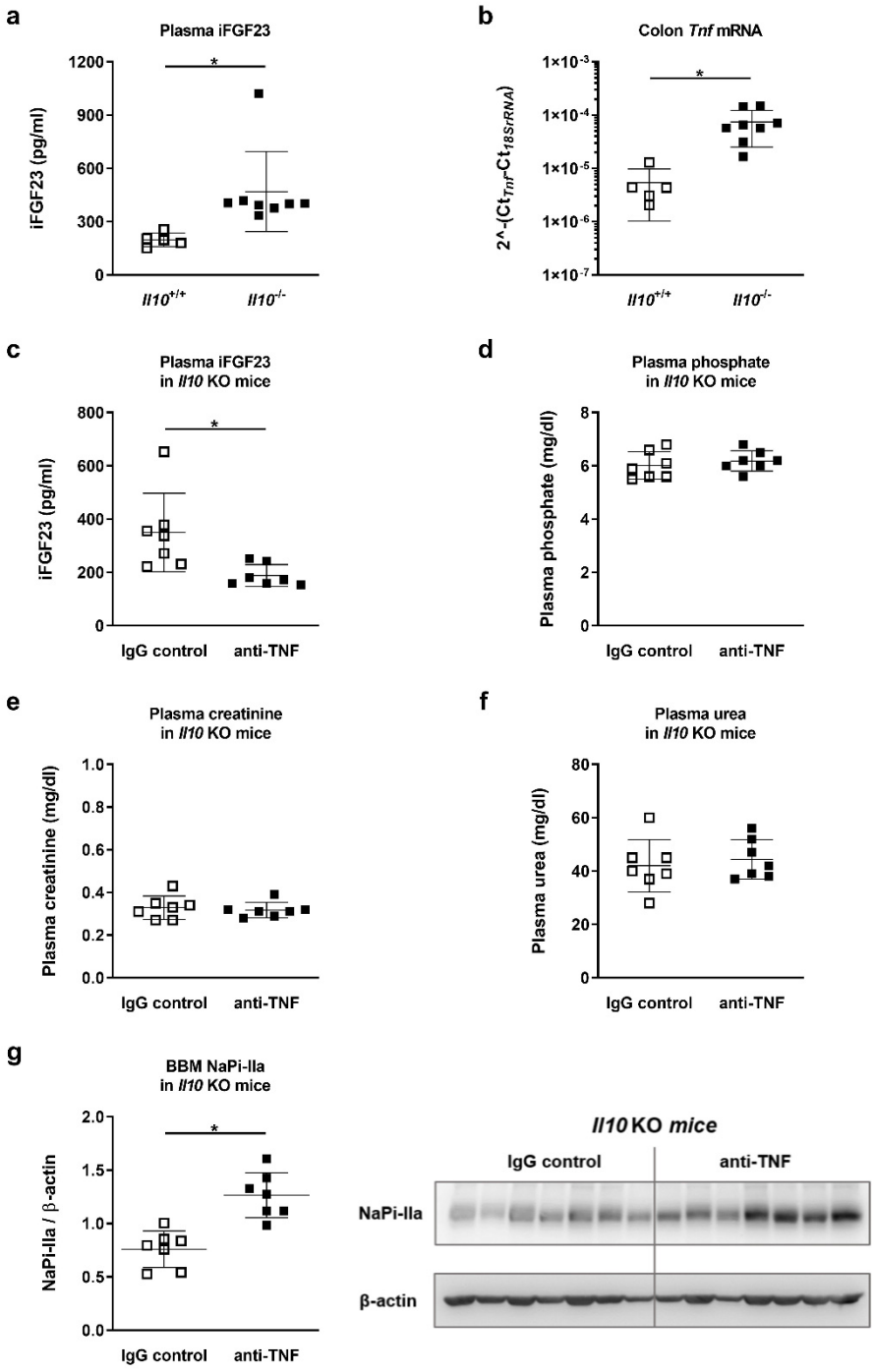
# Figure 4


a
Plasma iFGF23
iFGF23 (pg/ml)
vehicle
TNF
b
Plasma TNF
Plasma TNF (pg/ml)
ND
c
Plasma phosphate
Plasma phosphate (mg/dl)
d
Plasma creatinine
Plasma creatinine (mg/dL)
e
Plasma urea
Plasma urea (mg/dl)
f
FEPi
FEPi (%)
g
Bone Fgf23 mRNA
h
Spleen Fgf23 mRNA
i
Thymus Fgf23 mRNA
j
Bone marrow Fgf23 mRNA
Vehicle
k
Fgf23
Fgf23 fold change
VitD
l
Dmp1
Dmp1 fold change
m
Galnt3
Galnt3 fold change
n
Nurr1
Nurr1 fold change

# Figure 5



# Figure 6



## Supplementary Tables

**Supplementary Table 1** First quartile, median, mean and third quartile of continuous variables in the SKIPOGH population after full exclusion criteria applied (429 subjects).

| Variable | 1st Qu. | Median | Mean | 3rd Qu. |
|---|---|---|---|---|
| Age (yr) | 29.7 | 46.6 | 45.7 | 57.9 |
| BMI (kg/m$^2$) | 21.8 | 24.3 | 24.6 | 26.7 |
| eGFR (CKD-EPI) (mL/min/1.73 m$^2$) | 87.1 | 98.4 | 98.1 | 110.1 |
| IFNγ (pg/ml) | 3.2 | 4.7 | 9.0 | 7.1 |
| IL-10 (pg/ml) | 0.2 | 0.3 | 0.5 | 0.4 |
| IL-1β (pg/ml) | 0.0 | 0.1 | 0.1 | 0.1 |
| IL-6 (pg/ml) | 0.4 | 0.6 | 0.9 | 0.8 |
| TNF (pg/ml) | 2.2 | 2.7 | 2.9 | 3.4 |
| iFGF23 (pg/ml) | 38.8 | 46.2 | 49.2 | 55.3 |
| cFGF23 (Ru/ml) | 63.7 | 79.4 | 97.3 | 103.4 |
| Ratio iFGF23 (pg/ml) / cFGF23 (Ru/ml) | 0.4 | 0.6 | 0.6 | 0.7 |
| Iron (µmol/l) | 13.9 | 17.3 | 18.7 | 22.5 |
| Transferrin (µmol/l) | 28.0 | 31.0 | 32.2 | 35.0 |
| Ferritin (µg/l) | 62.0 | 119.0 | 143.2 | 192.0 |
| Phosphate (mmol/l) | 0.9 | 1.0 | 1.0 | 1.1 |
| Calcium (mmol/l) | 2.2 | 2.3 | 2.3 | 2.3 |
| PTH (pg/ml) | 29.8 | 37.1 | 38.8 | 45.0 |
| 25(OH)D (nmol/l) | 33.0 | 44.0 | 47.4 | 61.0 |
| 1,25(OH)$_2$D (pmol/l) | 67.0 | 88.0 | 92.3 | 116.0 |

**SupplementaryTable 2** First quartile, median, mean and third quartile of continuous variables in the SKIPOGH population without drug intake criteria applied (790 subjects).

| Variable | 1st Qu. | Median | Mean | 3rd Qu. |
|---|---|---|---|---|
| Age (yr) | 32.3 | 48.7 | 47.9 | 61.8 |
| BMI (kg/m2) | 21.8 | 24.3 | 24.9 | 27.3 |
| eGFR (CKD-EPI) (mL/min/1.73 m²) | 84.7 | 96.9 | 96.0 | 108.3 |
| IFNγ (pg/ml) | 3.4 | 4.9 | 9.0 | 7.6 |
| IL-10 (pg/ml) | 0.2 | 0.3 | 0.5 | 0.4 |
| IL-1β (pg/ml) | 0.0 | 0.1 | 0.1 | 0.1 |
| IL-6 (pg/ml) | 0.4 | 0.6 | 0.9 | 0.9 |
| TNF (pg/ml) | 2.2 | 2.8 | 3.1 | 3.6 |
| iFGF23 (pg/ml) | 39.0 | 47.2 | 50.6 | 57.6 |
| cFGF23 (Ru/ml) | 64.7 | 81.4 | 103.2 | 107.9 |
| Ratio iFGF23 (pg/ml) / cFGF23 (Ru/ml) | 0.4 | 0.6 | 0.6 | 0.7 |
| Iron (µmol/l) | 13.6 | 17.3 | 18.3 | 22.0 |
| Transferrin (µmol/l) | 28.0 | 31.0 | 32.0 | 35.0 |
| Ferritin (µg/l) | 62.2 | 115.5 | 142.4 | 180.1 |
| Phosphate (mmol/l) | 0.9 | 1.0 | 1.0 | 1.2 |
| Calcium (mmol/l) | 2.2 | 2.3 | 2.3 | 2.3 |
| PTH (pg/ml) | 29.8 | 37.4 | 39.4 | 45.8 |
| 25(OH)D (nmol/l) | 33.0 | 45.0 | 48.0 | 61.0 |
| $1,25(OH)_2D$ (pmol/l) | 68.0 | 88.0 | 92.8 | 115.0 |

**Supplementary Table 3** Exclusion criteria for SKIPOGH subjects

| Exclusion criteria | | N |
|---|---|---|
| Total SKIPOGH subjects | | 1131 |
| Incomplete data | - | 261 |
| No TNF detected | - | 40 |
| Odd iFGF23/ cFGF23 ratio | - | 40 |
| Drugs | - | 361 |
| Total study subjects | | 429 |

**Supplementary Table 4** Drug types which led to exclusion of SKIPOGH subjects for data analysis. The table shows 491 drug intake entries (N) for 361 subjects. Each entry represents each time a drug appears in the data base of 429 subjects after all exclusion criteria applied.

| Drug type | N |
|---|---|
| Interaction with Ca, Mg or Pi | 41 |
| Interaction with iron | 6 |
| Diuretic or diuretic action | 54 |
| Interaction with inflammation | 390 |

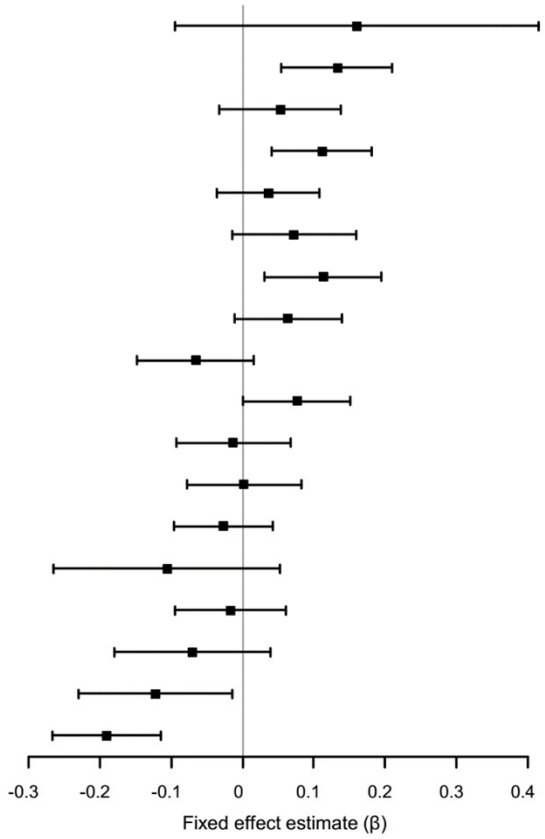
**Supplementary Table 5** Primer and probe sequences used for qPCR

| Gene | Type | Sequence |
|---|---|---|
| Mouse *Pkd1* | Fwd | 5'-GGCCAACCTCTCCTCAGTATC-3' |
| | Rev | 5'-GAAGGGTACTGCTGCCACA-3' |
| | Probe | 5'-CTGTGGTGAGGAATATGTCGCCTGC-3' |
| Mouse *Fgf23* | Fwd | 5'-TCGAAGGTTCCTTTGTATGGAT-3' |
| | Rev | 5'-AGTGATGCTTCTGCGACAAGT-3' |
| | Probe | 5'-TTTTTGGATCGCTTCACT-3' |
| Mouse *Fgf23* | Fwd | 5'-GTATGGATCTCCACGGCAAC-3' |
| | Rev | 5' AGTGATGCTTCTGCGACAAGT-3' |
| | Probe | 5'-TTTTTGGATCGCTTCACTTCAGCCC-3' |
| Mouse *Fgf23* | Fwd | 5'-GACCAGCTATCACCTACAGATCCA-3' |
| | Rev | 5' CGGCGTCCTCTGATGTAATCA-3' |
| Mouse *Galnt3* | Fwd | 5'-GAGAAAGAGCGAGGGGAAAC-3' |
| | Rev | 5'-GTGGACCATGCTTCATTGTG-3' |
| | Probe | 5'-ACACCCGACCACCTGAATGTATTGAAC-3' |
| Mouse *Dmp1* | Fwd | 5'-TCTCCCAGTTGCCAGATACC-3' |
| | Rev | 5'-TCTCCAGATTCACTGCTGTCC-3' |
| | Probe | 5'-CTCTGAAGAGAGGACGGGTGATTTGG-3' |
| Mouse *Runx2* | Fwd | 5'-CCCTGAACTCTGCACCAAGT-3' |
| | Rev | 5'-AGTGGATGGATGGGGATGT-3' |
| | Probe | 5'-TCAGATTACAGATCCCAGGCAGGCA-3' |
| Mouse *Nurr1* | Fwd | 5'-CATCGACATTTCTGCCTTCTC-3' |
| | Rev | 5'-CTTCCACTCTCTTGGGTTCCT-3' |
| | Probe | 5'-TGCCCTGGCTATGGTCACAGAGAGA-3' |
| Mouse *Tnf* | Fwd | 5'-CAGACCCTCACACTCAGATCATCT-3' |
| | Rev | 5'-CCTCCACTTGGTGGTTTGCT-3' |
| | Probe | 5'-ATTCGAGTGACAAGCCTGTAGCCCACGT-3' |
| Mouse *Tgfb* | Fwd | 5'-AGAGGTCACCCGCGTGCTA-3' |
| | Rev | 5'-GCTTCCCGAATGTCTGACGTA-3' |
| | Probe | 5'-ACCGCAACAACGCCATCTATGAGAAAACC-3' |
| Mouse *Car9* | Fwd | 5'-GCACCTCAGTACTGCTTTCTCC-3' |
| | Rev | 5'-TTCCTCCGAGATTTCTTCCA-3' |
| | Probe | 5'-CCTTTCTGCAGGAGAGCCCAGAAGA-3' |
| Mouse *Phd2* | Fwd | 5'-TGGGCAACTACAGGATAAACG-3' |
| | Rev | 5'-TGTCACGCATCTTCCATCTC-3' |
| | Probe | 5'-CGAAAGCCATGGTTGCTTGTTACCC-3' |

**a**

**Without exclusion by drug intake**

| | β | SE | t-value | p-value |
|---|---|---|---|---|
| Intercept | 0.160 | 0.13 | 1.232 | 0.219 |
| 25(OH)D | 0.133 | 0.039 | 3.373 | 0.001 |
| TNF | 0.053 | 0.043 | 1.229 | 0.220 |
| Calcium | 0.111 | 0.035 | 3.128 | 0.002 |
| Iron | 0.036 | 0.037 | 0.976 | 0.329 |
| IL-6 | 0.072 | 0.044 | 1.645 | 0.100 |
| PTH | 0.113 | 0.041 | 2.725 | 0.007 |
| Transferrin | 0.064 | 0.038 | 1.674 | 0.095 |
| BMI | -0.066 | 0.042 | -1.575 | 0.116 |
| Phosphate | 0.076 | 0.038 | 1.976 | 0.049 |
| IFNγ | -0.013 | 0.041 | -0.309 | 0.758 |
| IL-10 | 0.002 | 0.041 | 0.054 | 0.957 |
| IL-1b | -0.026 | 0.035 | -0.745 | 0.456 |
| Sex | -0.106 | 0.081 | -1.315 | 0.189 |
| Ferritin | -0.017 | 0.039 | -0.427 | 0.669 |
| Age | -0.070 | 0.056 | -1.258 | 0.209 |
| eGFR | -0.122 | 0.054 | -2.245 | 0.025 |
| 1,25(OH)2D | -0.190 | 0.039 | -4.935 | 0 |




**b**

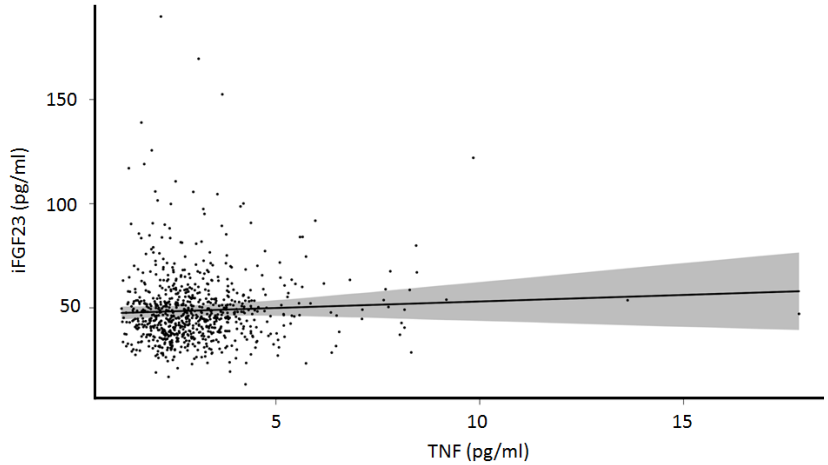
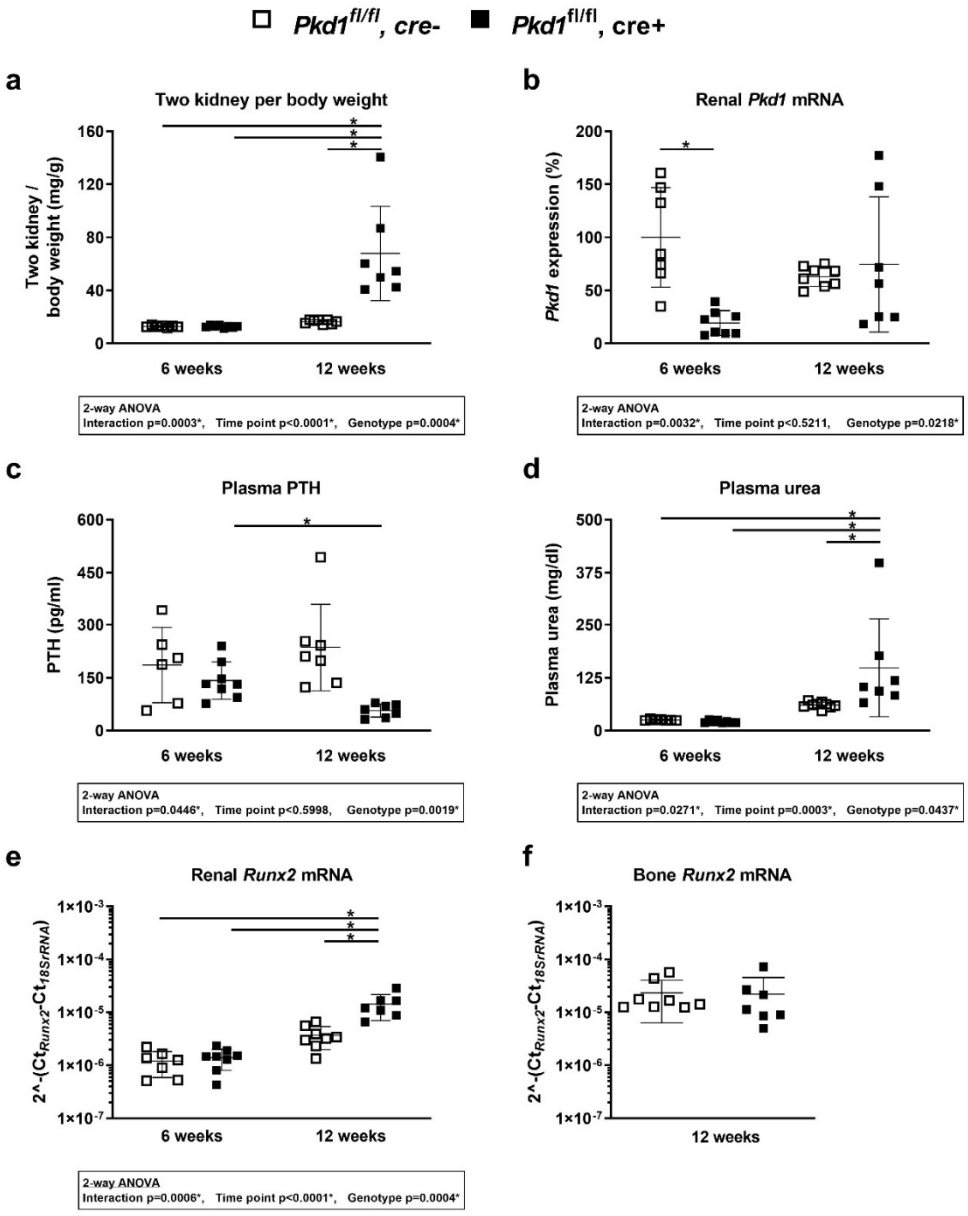



**Figure S1**

**Identification of plasma iFGF23 predictors in a human cohort.** (a) Forest plot showing the fixed effects calculated for all predictors used in the mixed linear model for the subpopulation of 790 participants without drug intake criteria applied. Fixed effect estimates (β), standard error, ratio between the estimates and their standard errors (t-value), and associated p-value from a t-distribution. (b) Association between plasma TNF and iFGF23 in the SKIPOGH cohort in a subpopulation of 790 participants after all the exclusion criteria applied. The regression line and confidence band were obtained from the linear mixed model containing all the predictors.




## Figure S2

**Baseline characteristics of *Pkd1* KO mice.** Two-kidney per body weight ratio (a), *Pkd1* gene expression normalized to *Pkd*$^{fl/fl}$, *cre*- mice (6 weeks) (b), plasma iFGF23 (c), plasma PTH (d) and plasma urea (d) as well as *Runx-2* (e, f) mRNA expression relative to 18SrRNA in kidney and bone in *Pkd1*$^{fl/fl}$, *cre*- (white squares) and *Pkd1*$^{fl/fl}$, *cre*+ (black squares) animals at the age of 6 and 12 weeks. Two-way ANOVA with Bonferroni correction (a-d, f) or unpaired t-test (e), * p<0.05.

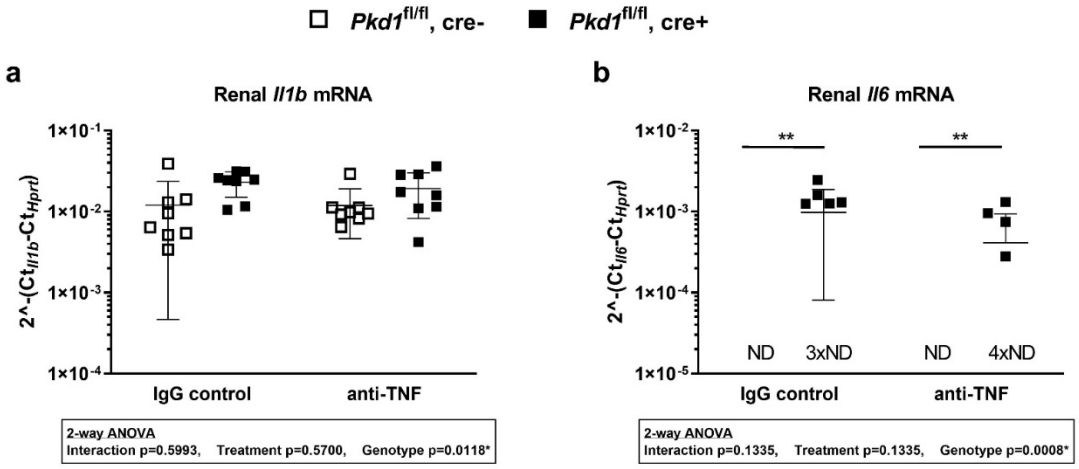
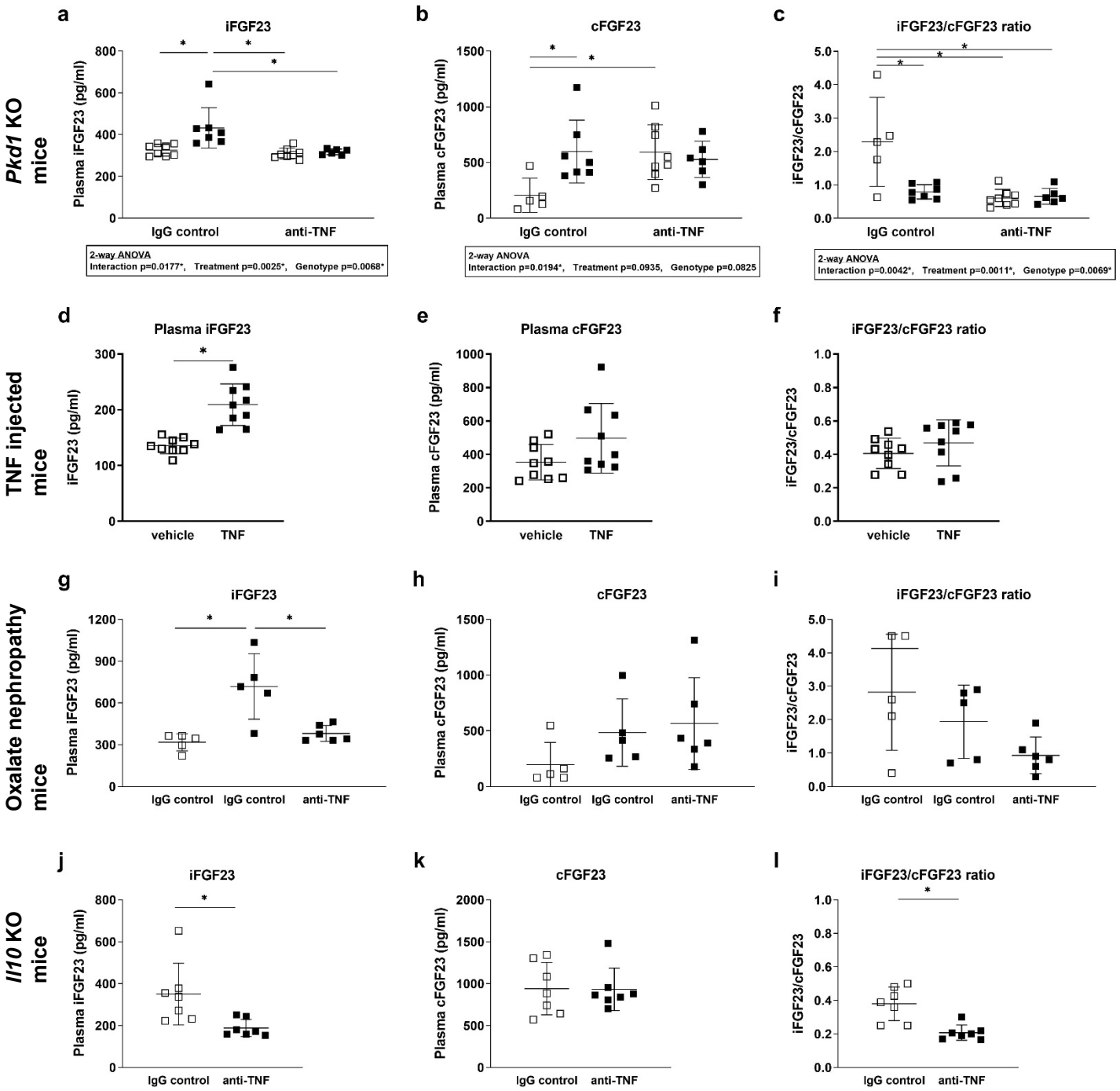



**Figure S3**

**Expression of inflammation markers in the kidney of *Pkd1* KO mice.** Renal *Il1b* (a), and renal *Il6* (b) mRNA expression relative to 18SrRNA in *Pkd1$^{fl/fl}$, cre-* (white squares) and *Pkd1$^{fl/fl}$, cre+* (black squares) animals at the age of 6 and 12 weeks. Two-way ANOVA with Bonferroni correction, * $p<0.05$.




## Figure S4

**Comparison of plasma iFGF23, cFGF23 and iFGF23 to cFGF23 ratio.** Plasma iFGF23 (a, d, g, j), plasma cFGF23 (b, e, h, k) and iFGF23/cFGF23 ratio (c, f, i, l) in *Pkd1* KO mice (*Pkd1*$^{fl/fl}$, *cre*- (white squares) and *Pkd1*$^{fl/fl}$, *cre*+ (black squares)), TNF injected mice (2ug/day for two consecutive days), oxalate nephropathy mice (control diet (white squares) and oxalate nephropathy (black squares)). Two-way ANOVA with Bonferroni correction (a-c), One-way ANOVA with Bonferroni correction (g-i) or unpaired t-test (d-f, j-l), * $p<0.05$.

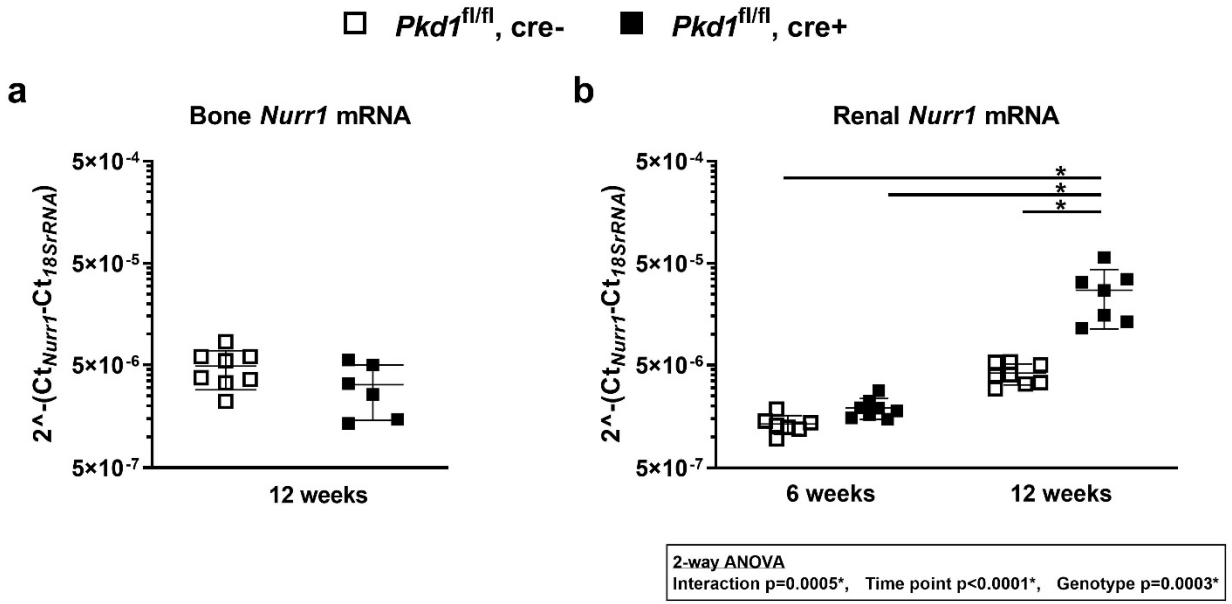



**Figure S5**

**Bone and renal *Nurr1* mRNA expression in *Pkd1* KO mice.** *Nurr1* mRNA expression relative to 18SrRNA in kidney (a) and bone (b) in *Pkd1*$^{fl/fl}$*, cre*- (white squares) and *Pkd1*$^{fl/fl}$*, cre*+ (black squares) animals at the age of 6 and 12 weeks. Unpaired t-test (a), or Two-way ANOVA with Bonferroni correction (b), * $p<0.05$.

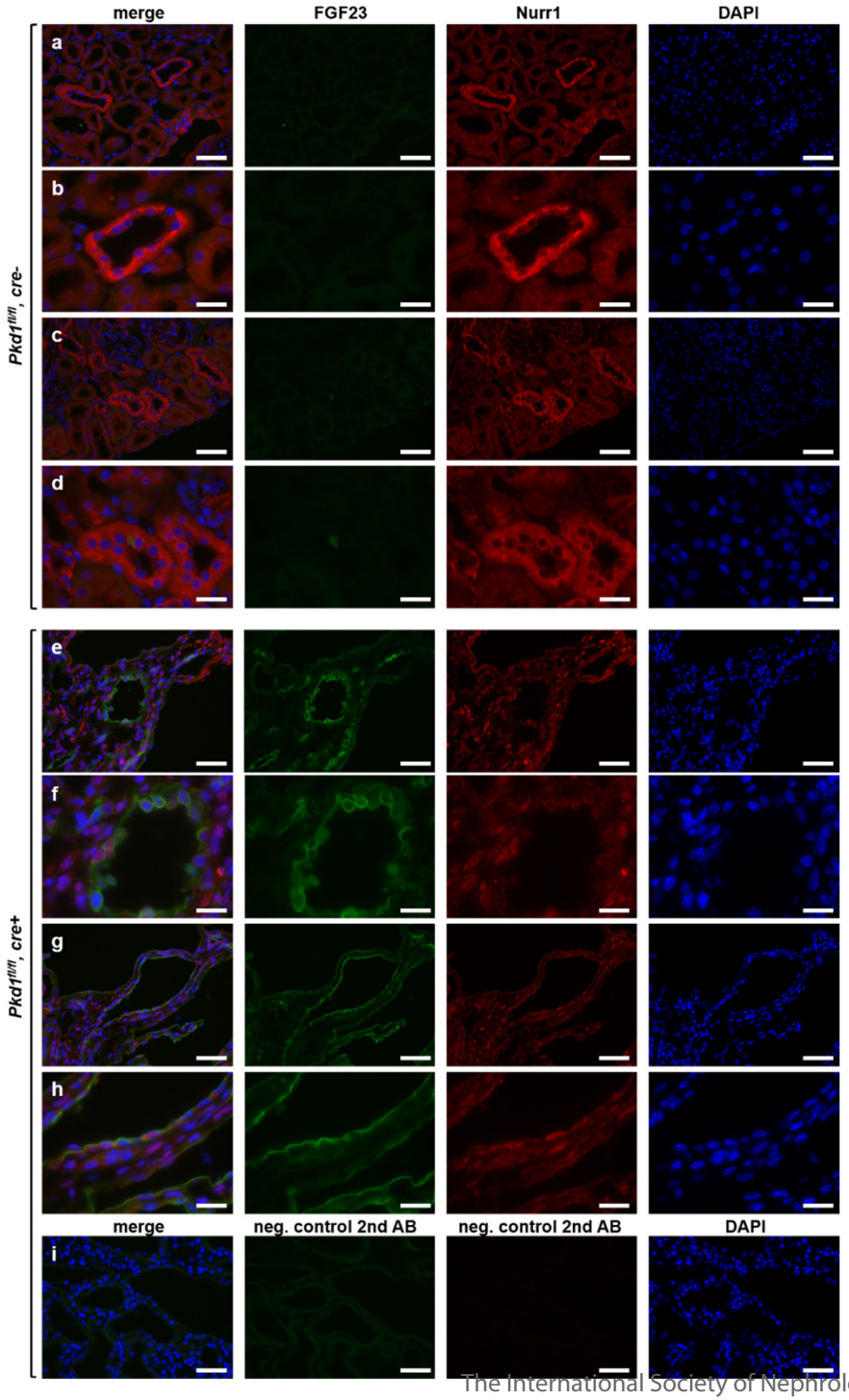
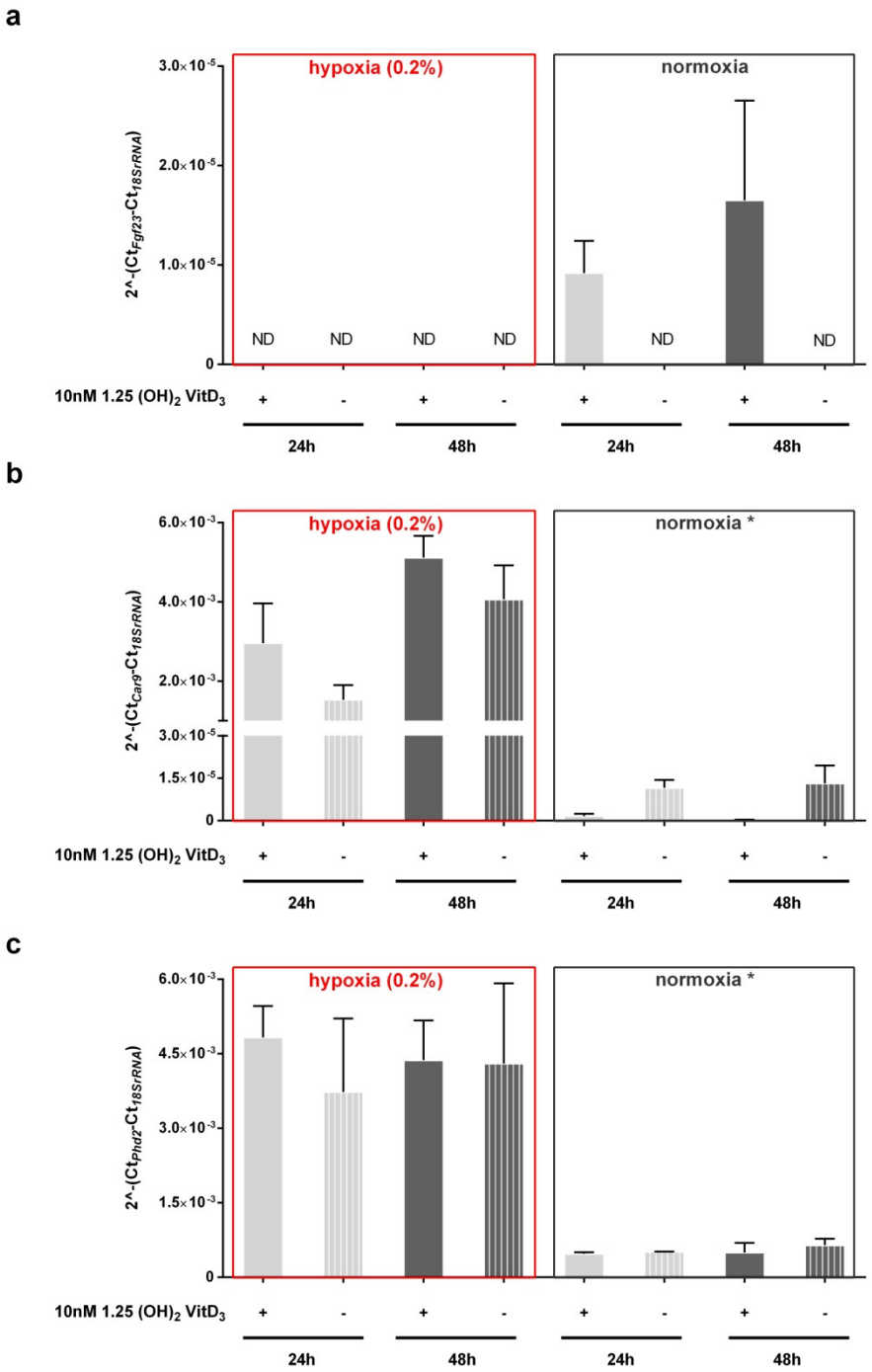



**Figure S6**

**Colocalization of Nurr1 and FGF23 in *Pkd1* kidneys.** Immunofluorescence staining for FGF23 (green), Nurr1 (red), and DAPI (blue, cell nuclei) merge and single channels in kidneys of 12 weeks old *Pkd1*$^{fl/fl}$, *cre*- (a-d), *Pkd1*$^{fl/fl}$, *cre*+ (e-h) and *Pkd1*$^{fl/fl}$, *cre*+ incubated with secondary antibodies alone (i). Original magnification (a, c, e, g) 400x (scale bar 50 μm) and (b, d, f, h) 1000x (scale bar 20 μm).




## Figure S7

**1,25 $(OH)_2$ vitamin $D_3$ dependent *Fgf23* expression in MC3T3-E1 cells under hypoxic conditions.** MC3T3-E1 cells were differentiated for two weeks along the osteogenic lineage. Subsequently, cells were supplemented for 24 or 48 hours with 10nM 1,25 $(OH)_2$ vitamin $D_3$ and incubated under hypoxic (0.2% oxygen) or normoxic conditions. *Fgf23* (a), *Car9* (b), and *Phd2* (c) mRNA expression relative to 18SrRNA. Mean ±SD; 3 independent experiments; ANOVA with Bonferroni correction, * $p<0.05$.

# Antibody mediated TNF ~~stimulates~~neutralization decreases FGF23 levels in animal models of chronic kidney disease and non-renal inflammation

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# Abstract

Fibroblast growth factor 23 (FGF23) regulates phosphate homeostasis and its early rise in patients with chronic kidney disease (CKD) is ~~directly linked to~~independently associated with all-cause mortality. Since inflammation is characteristic for CKD and has been associated with plasma FGF23 we examined whether inflammation directly stimulates FGF23. In a population-based cohort, plasma tumor necrosis factor (TNF) was the only inflammatory cytokine that independently and positively correlated with plasma FGF23. Mouse models of CKD showed signs of renal inflammation, renal FGF23 expression and elevated systemic FGF23. Renal FGF23 expression coincided with expression of the orphan nuclear receptor Nurr1 regulating FGF23 in other organs. Antibody-mediated neutralization of TNF normalized plasma FGF23 and ectopic renal *Fgf23* expression. Conversely, TNF administration to control mice increased plasma FGF23 without altering plasma phosphate. Similarly, in ~~*Il-10*~~*Il10* deficient mice with inflammatory bowel disease and normal kidney function, FGF23 was elevated and normalized upon TNF neutralization. In conclusion, the inflammatory cytokine TNF contributes to elevated systemic FGF23 levels and triggers also ectopic renal *Fgf23* expression in CKD animal models.



# Keywords

Fibroblast growth factor 23 (FGF23), tumor necrosis factor (TNF), chronic kidney disease (CKD), inflammation, cytokine, inflammatory bowel disease, bone.

# INTRODUCTION

Chronic kidney disease (CKD) causes a severe disturbance of mineral metabolism, one of the leading factors for morbidity and mortality in patients with end stage renal disease (ESRD) [1, 2]. Fibroblast growth factor 23 (FGF23) increases early during CKD progression and is required to maintain serum phosphate levels while kidney function declines [3]. In CKD patients, high FGF23 levels are associated with an increased risk of mortality independent of plasma phosphate [4]. FGF23 promotes left ventricular hypertrophy in rodents [5] and elevated FGF23 is a risk factor in the general population for all-cause and cardiovascular mortality [6].

FGF23 is critical for the regulation of phosphate homeostasis and vitamin $D_3$ metabolism [7]. The main target organ of FGF23 is the kidney where FGF23 binds together with αKlotho to FGF receptors and inhibits phosphate reabsorption and decreases 1,25-$(OH)_2$ vitamin $D_3$ (1,25$(OH)_2$D) [8, 9]. FGF23 levels are regulated by a variety of stimuli including calcitriol, PTH, insulin, aldosterone, erythropoietin, and adipokinines [8, 10-13]. Moreover, FGF23 may be linked to inflammation. In the *Chronic Renal Insufficiency Cohort* elevated FGF23 is independently associated with higher IL-6 and TNF [14] and also in a smaller cohort with only 103 CKD patients, RANTES and IL-12 associated with higher FGF23 [15]. The association between FGF23 and inflammation markers is not limited to CKD. *The Reasons for Geographic and Racial Differences in Stroke* study found a positive correlation of FGF23 with IL-6 and IL-10 in a non-CKD population [16]. Children during an acute phase of inflammatory bowel disease (IBD) had elevated FGF23 that normalized in the remission phase [17]. Furthermore, chondrocytes from patients with osteoarthritis have elevated *Fgf23* gene expression [18]. Microarray data from mouse models with FGF23 excess (*Col4a3* KO, *Hyp*, and *Fgf23* transgenic mice) show an activation of genes important in the regulation of the inflammatory response such as transforming growth factor beta (TGFβ), tumor necrosis factor (TNF) and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) [19]. Further, inflammatory stimuli and the hypoxia inducible transcription factor HIF-1 enhance FGF23 expression: TNF and TGFβ2

increases FGF23 expression in bone cells *in vitro* and HIF-1, interleukin-1 beta (IL-1β), lipopolysaccharide (LPS) increase FGF23 expression *in vitro* and *in vivo* [20-25]. Also, in an obesity induced model, TNF is necessary for the increase in FGF23 levels [26]. Some inflammatory stimuli, including TNF, may act on *Fgf23* transcription via a 16 kb enhancer element [27]. Moreover, in the folic-acid induced AKI model as well as in the adenine CKD model, genetic ablation of Il-6 reduced the increase in FGF23 [28]. Thus, inflammatory cytokines may play an important role at least in the early phase of CKD to induce FGF23. However, whether TNF is a critical player has not been demonstrated.

Here, we investigated the association between inflammatory cytokines with plasma FGF23 in a population-based cohort and evaluated the effect of TNF on the regulation of plasma FGF23 in CKD animal models and in a non-renal inflammation model. Furthermore, we evaluated the role of hypoxia on *Fgf23* gene expression. Our results demonstrate a critical role for TNF to stimulate FGF23 in models of renal and non-renal inflammatory diseases.

# Results

## Plasma TNF positively correlated with intact FGF23 in the SKIPOGH population based cohort

The *Swiss Kidney Project on Genes in Hypertension* (SKIPOGH) is a family and population-based, multicenter, cross-sectional study including 1131 subjects randomly selected [29]. We assessed the relationship between plasma intact FGF23 (iFGF23) and parameters of phosphate metabolism, inflammatory cytokines, and iron metabolism while considering familial correlation. Participants with drugs interacting with calcium, magnesium and phosphate metabolism, inflammation and iron metabolism or have diuretic action were excluded. Based on a linear mixed model with family as random effect, 1,25-((OH)$_2$ vitamin D$_3$2D, 25-(OH) vitamin D$_3$, (25(OH)D), TNF, and calcium, and iron are showed the highest fixed effects and were considered significant predictors of plasma iFGF23 while holding all the other variables constant (Figure 1 and Table S1).). The standard deviation of the random effect was low compared to the standard deviation of the residuals (0.26 vs 0.93), which means that most of the variation in iFGF23 levels was due to the fixed effects (i.e. hormones, cytokines, etc.). There was no correlation between plasma iFGF23 and plasma phosphate, PTH, or eGFR. Besides TNF, no other inflammatory cytokine such as interferon gamma (IFNγ), IL-1β, IL-6, or IL-10 correlated with plasma iFGF23.

Iron metabolism affects plasma FGF23 in mice [21, 24, 30]. In the SKIPOGH cohort iron is associated with plasma iFGF23, however, there was no correlation between plasma iFGF23 and ferritin and transferrin. Plasma iFGF23 was not dependent on age, body mass index, or sex.

We also analyzed the cohort without applying exclusion criteria based on drugs. TNF remained together with 1,25-((OH)$_2$ vitamin D$_3$2D, 25-((OH) vitamin D$_3$D, and calcium remained as a predictorpredictors of iFGF23 while phosphate, PTH and eGFR arose as

additional predictors of iFGF23 (Figure S1 ~~and Table S2).~~). The TNF effect on iFGF23 is reduced in this population. . First quartile, median, mean and third quartile of continuous variables in the SKIPOGH population with and without drug intake criteria applied are listed in ~~the~~ Tables S1 and S2.

## Inflammation in kidneys of *Pkd1* conditional KO mice

TNF is increased in CKD patients, stimulates FGF23 expression in an osteocyte cell line, and was the only inflammatory cytokine associated with iFGF23 in the SKIPOGH cohort [14, 22, 31, 32]. Thus, we tested in two CKD mouse models whether TNF contributes to the rise of iFGF23 during the early phase of kidney disease. First, slowly progressing polycystic kidney disease (PKD) was induced in ~~*Pkd 1*~~*Pkd1* conditional KO mice [33]. Kidney function and two-kidney per body weight ratio were similar in 6 week old mice whereas kidney function was decreased and two-kidney per body weight ratio was increased in 12 week old *Pkd1*, *cre*+ mice (Figure S2). At week 6, iFGF23, TmP/GFR as well as renal *Tnf* and *Tgfb* mRNA expression were similar in *Pkd1*$^{fl/fl}$, *cre*- and *Pkd1*$^{fl/fl}$, *cre*+ ~~animals~~mice (Figure 2 a ~~and b-~~ d). Progression of kidney disease ~~correlated with~~was accompanied by increased plasma iFGF23, decreased TmP/GFR as well as increased *Tnf* and *Tgfb* mRNA expression in *Pkd1*$^{fl/fl}$, *cre*+ mice (Figure 2 a ~~and b-~~ d). TNF binding to TNF receptors activates the NFκB signaling pathway. The ratio of phospho-NFκB p65 to total NFκB p65 protein in the nuclear fraction of total kidney was significantly elevated in *Pkd1*$^{fl/fl}$, *cre*+ mice (Figure ~~2c~~2 e). Increased renal inflammatory cytokines in 12 week old *Pkd1*$^{fl/fl}$, *cre*+ mice were paralleled by the appearance of renal *Fgf23* expression and by the upregulation of the osteogenic marker gene *Runx2* in the kidney (Figure ~~S3 a~~2 f and ~~c~~S2 e). Bone *Fgf23* and *Runx2* mRNA expression were unchanged (Figure ~~S3 b~~2 g and ~~d~~S2 f).

## TNF blockade in *Pkd1* conditional KO mice suppressed FGF23

The effect of acute TNF blockade on FGF23 expression in PKD kidneys and on plasma iFGF23 was investigated. We injected intraperitoneally (i.p.) a single dose of 0.5 mg anti-TNF antibody or isotypic IgG control into 12 week old *Pkd1*$^{fl/fl}$, *cre*+ and *Pkd1*$^{fl/fl}$, *cre*- mice. After 24 hours, anti-TNF treated mice had a significant reduction of plasma TNF compared to the IgG control treated mice confirming the efficacy of the anti-TNF antibody (Figure 3 a). There was no difference in plasma TNF between IgG control treated *Pkd1*$^{fl/fl}$, *cre*+ and *Pkd1*$^{fl/fl}$, *cre*- mice. Importantly, elevated plasma iFGF23 in *Pkd1*$^{fl/fl}$, *cre*+ mice was normalized by anti-TNF but not IgG control treatment (Figure 3 b). ~~There was no change in plasma phosphate or urea levels (Figure 3 c and d).~~Plasma C-terminal FGF23 (cFGF23) was increased in IgG control treated *Pkd1*$^{fl/fl}$, *cre*+ and anti-TNF treated *Pkd1*$^{fl/fl}$, *cre*- compared to IgG control treated *Pkd1*$^{fl/fl}$, *cre*- mice consequently the iFGF23/cFGF23 ratio was elevated in IgG control treated *Pkd1*$^{fl/fl}$, *cre*- mice (Figure S4 a – c). There was no change in plasma phosphate and urea. ~~as well as t~~(Figure 3 c and d). The abundance of the sodium dependent phosphate co-transporter NaPi-IIa in~~at~~ the brush border membrane (BBM) ~~increased in *Pkd1*$^{fl/fl}$, *cre*+ mice when treated with anti-TNF antibodies~~showed a trend to increase in *Pkd1*$^{fl/fl}$, *cre*+ mice when treated with anti-TNF antibodies – (Figure 3 ~~c -~~ e). In *Pkd1*$^{fl/fl}$, *cre*+ mice, TNF neutralization decreased ectopic renal *Fgf23* mRNA expression while *Fgf23* mRNA expression in bone (Figure 3 ~~e~~f and ~~f~~g) and ~~renal~~ *Tnf* ~~or~~, and *Tgfb* mRNA expression in kidney (Figure 3 ~~g and~~ h and i) were unchanged. The mRNA expression of the inflammatory cytokines *Il1b* and *Il6* was elevated in PKD kidneys but did not ~~affected.~~change with anti-TNF treatment (Figure S3). ~~Mace et al. showed that renal Fgf23 expression did not contribute to total circulating FGF23 levels [34]. Here, TNF blockade also reduced renal *Fgf23* expression in *Pkd1*$^{fl/fl}$, *cre*+ mice. TNF itself may trigger renal *Fgf23* expression which in turn may promote further local inflammation and fibrosis [35, 36].~~ The orphan nuclear receptor Nurr1 is downstream of TNF signaling and activates *Fgf23* mRNA expression in rat osteosarcoma cells upon PTH treatment [37, 38]. *Nurr1* mRNA was detected in mouse kidney and bone

(Figure ~~S4 a and b~~S5). In the kidney of 12 week old *Pkd1*$^{fl/fl}$*, cre+* mice, *Nurr1* mRNA expression was upregulated and Nurr1 protein was predominantly localized in the cell nucleus compared to *Pkd1*$^{fl/fl}$*, cre-* mice where Nurr1 was mainly distributed in the cytoplasm (Figure ~~S5~~S6). Further, nuclear Nurr1 staining in *Pkd1*$^{fl/fl}$*, cre+* mice was often co-localized with FGF23. ~~We assessed the relationship between renal *Fgf23* and *Nurr1* expression in C57Bl/6J mice undergoing unilateral ureteral ligation (UUO). Fourteen days after surgery, we detected *Fgf23* mRNA expression only in the UUO kidney but not in the contralateral control kidney. Ectopic *Fgf23* expression was paralleled by higher *Tnf* and *Nurr1* mRNA expression (Figure S4 c - e).~~

## TNF but not hypoxia increased FGF23 levels

We evaluated the effect of systemic TNF administration on plasma iFGF23. ~~and~~T~~t~~herefore we injected wild type mice for two consecutive days with ~~a single dose of~~ 2 µg recombinant mouse TNF. After 48-hours, plasma iFGF23 increased while cFGF23 and the iFGF23/cFGF23 ratio were unchanged (Figure 4a and S4 d – f). Furthermore plasma TNF and fractional excretion of phosphate~~,~~ increased, plasma urea decreased while plasma phosphate and creatinine~~, and urea~~ levels were unchanged (Figure 4 ~~a - d).~~b - f). In bone and spleen *Fgf23* mRNA expression decreased in TNF injected compared to vehicle injected mice whereas *Fgf23* mRNA expression in thymus and bone marrow was unchanged (Figure 4 g -j). We cultured primary osteocytes from tibias and femurs of mice [39, 40] for 2 weeks before being supplemented for 24 hours either with 10 ng/ml TNF or 10 nM 1,25-~~(~~(OH)$_2$ ~~vitamin D$_3$~~D. TNF as well as 1,25-~~(~~(OH)$_2$ ~~vitamin D$_3$~~D increased *Fgf23* mRNA expression (Figure 4 ~~e~~k). TNF and 1,25-~~(~~(OH)$_2$ ~~vitamin D$_3$~~D decreased the expression of *Dmp1* (Figure 4 ~~f~~l). Dmp1 inhibits *Fgf23* gene expression and loss of *DMP1* in patients causes hypophosphatemic rickets due to high FGF23 levels [41]. TNF but not 1,25-~~(~~(OH)$_2$ ~~vitamin D$_3$~~D increased the expression of *Galnt3* and *Nurr1* (Figure 4 ~~g~~m and ~~h~~n). Galnt3 mediates O-glycosylation of FGF23 preventing proteolytic

cleavage of FGF23 [42, 43]**Error! Reference source not found.Error! Reference source not found.**.

CKD kidneys are commonly affected by hypoxia [44, 45] which was recently suggested to stimulate FGF23 expression through the hypoxia inducible transcription factor HIF-1 [20, 21, 24]. We studied in MC3T3-E1 mouse preosteoblasts the effect of hypoxia on *Fgf23* gene expression. MC3T3-E1 did not display intrinsic *Fgf23* expression. Nevertheless, after 2 weeks osteogenic differentiation of MC3T3-E1, *Fgf23* mRNA expression was induced by 10 nM 1,25-~~((~~(OH)$_2$ ~~vitamin D$_3$~~2D. 1,25-~~((~~(OH)$_2$ ~~vitamin D$_3$~~2D-induced *Fgf23* mRNA expression was completely repressed by hypoxic conditions (0.2% $O_2$) for 24 or 48 hours and hypoxia alone failed to trigger *Fgf23* expression (Figure ~~S6~~S7 a). The upregulation of the HIF-1 target genes carbonic anhydrase 9 (*Car9*) and prolyl hydroxylase domain containing protein 2 (*Phd2*) confirmed the presence of hypoxia (Figure ~~S6~~S7 b and c). Similarly, hypoxia had no effect on *Fgf23* mRNA expression in U2OS rat osteosarcoma and primary osteoblast cells (data not shown). We analyzed also kidneys of von Hippel-Lindau (*Vhl*) KO animals [46]. Lack of VHL prevents HIF hydroxylation and degradation and activates hypoxia sensitive genes [47]. Neither the kidneys of *Vhl* KO animals nor primary kidney cells lacking *Vhl* [48] expressed any detectable *Fgf23* (data not shown).

## TNF blockade lowers FGF23 levels in mouse models of oxalate nephropathy and colitis

We expanded our observations to another CKD mouse model, the oxalate nephropathy model in order to test for the relationship between TNF and FGF23 in a non-genetically modified mouse model and during early stages of kidney disease [49]. After induction of oxalate nephropathy, 48 hours prior to sacrifice, mice received a single i.p. injection of 0.5 mg anti-TNF or isotypic IgG control antibodies. IgG injected oxalate nephropathy mice had elevated plasma iFGF23 compared to control mice and TNF blockade normalized the elevated plasma iFGF23 in oxalate nephropathy mice (Figure 5 a). Plasma cFGF23 and iFGF23/cFGF23 did not differ between the groups (Figure S4 g –

i). Plasma TNF was significantly reduced in the anti-TNF treated groups confirming the efficacy of the anti-TNF antibody (Figure 5 b). There was no difference in plasma TNF between IgG control treated oxalate nephropathy and control mice. Renal *Tnf* mRNA expression showed a clear trend to increase in oxalate nephropathy mice and was not affected by the anti-TNF antibody (Figure 5 c). There was no change in plasma phosphate and urine phosphate per urine creatinine ratio while the renal function parameters plasma creatinine and urea showed a trend to increase in the oxalate nephropathy mice (Figure 5 d – fg).

To demonstrate that TNF regulates plasma iFGF23 independent from impaired kidney function, we analyzed a non-renal inflammation model, the *Il-10Il10* KO mouse developing spontaneously colitis [50]. Twelve to fourteen weeks old *Il-10Il10* KO mice had elevated plasma iFGF23 and increased colon *Tnf* mRNA expression (Figure 6 a and b). After 48 hours of a single i.p. injection of 0.5 mg anti-TNF or IgG control, anti-TNF treated *Il-10Il10* KO mice had reduced plasma iFGF23 compared to IgG treated animals withoutwhereas cFGF23 levels were similar (Figure 6 c and S4 k). There was a reduction in the iFGF23/cFGF23 ratio in anti-TNF treated *Il10* KO compared to IgG control treated *Il10* KO mice (Figure S4 j — l). Anti-TNF treatment had no effect on plasma phosphate levels (Figure 6 c and d) or kidney function parameters (Figure 6 e and f). But there was an increase in abundance of NaPi-IIa at the BBM in *Il10* KO mice treated with anti-TNF antibodies compared to IgG control mice (Figure 6 g)

# Discussion

We provide a novel explanation for high iFGF23 levels in patients with chronic kidney disease or inflammation of non-renal origin. Our data demonstrate that TNF is positively and independently associated with plasma iFGF23 in humans. We show that exogenous TNF stimulates iFGF23 expression both *in vivo* and *in vitro*. TNF neutralization suppresses plasma iFGF23 in two CKD mouse models and triggers renal *Fgf23* expression in PKD kidneys. TNF also contributes to high iFGF23 in a model of intestinal inflammation with normal kidney function.

In humans, TNF levels correlated with plasma iFGF23 ~~independent of drug intake~~ in the SKIPOGH multi-centric population based cohort. Dhayat et al. found in the same cohort associations between ~~C-terminal FGF23 (~~cFGF23~~)~~ and plasma phosphate, 1,25-~~((~~OH)$_2$ ~~vitamin D$_3$~~$_2$D, 25-~~((~~OH) ~~vitamin D$_3$~~D, the ratio of TmP/GFR, age, sex, and renal function. However, there are relevant differences between both analyses: 1) we have measured both the biologically active iFGF23 and the biologically inactive C-terminal fragment, while Dhayat et al. [51] used a method that detects ~~both~~ the sum of the intact form and the ~~biologically inactive~~ C-terminal fragment. 2) in addition to the subjects excluded by Dhayat et al. we excluded individuals taking drugs interacting with inflammation and subjects without complete data available for all variables. However, both analyses identified 1,25-~~((~~OH)$_2$ ~~vitamin D$_3$~~$_2$D and 25-~~((~~OH) ~~vitamin D$_3$~~D as strong predictors of FGF23 variation in the SKIPOGH population while the correlation of ~~iron,~~ PTH and eGFR in our study was dependent on drug exclusion criteria. The overall effect of TNF on iFGF23 may explain only a small part of the overall variability of iFGF23 in this cohort.

TNF increases in kidney disease and associates with CKD progression [14, 31, 32]. TNF stimulates *Fgf23* mRNA expression in an osteocyte-derived cell line [22] and may be involved in obesity induced increases in FGF23 [26]. We tested the relevance of FGF23 regulation by TNF in pathological situations such as kidney disease or colitis. We used two distinct CKD mouse models, the *Pkd1* conditional KO mouse and the oxalate

nephropathy model. PKD kidneys are affected by inflammation [52, 53] as confirmed by higher renal *Tnf* and *Tgfb* expression as well as enhanced NFκB subunit p65 phosphorylation. Similarly, in oxalate nephropathy the inflammasome is activated and various proinflammatory cytokines are released [49, 54]**Error! Reference source not found.**. Ectopic renal FGF23 gene and protein expression occurs in rodents with either diabetic nephropathy, PKD, or 5/6 nephrectomy [34, 55, 56]. The increase of renal *Tnf* and *Tgfb* mRNA expression in PKD kidneys was paralleled by the increase in plasma iFGF23 levels, and the appearance of renal *Fgf23* and *Runx-2* expression. Renal FGF23 production may promote inflammation and fibrosis in the affected kidney [35, 36, 57]. We did not detect any change in bone *Fgf23* mRNA expression or plasma TNF levels in both CKD models. Similarly, in *Col4a3* KO mice, another CKD model, the early rise in plasma FGF23 is not accompanied by increased *Fgf23* expression in bone [58]. TNF blockade in both CKD models normalized plasma iFGF23 levels without changes in plasma phosphate levels. In the PKD model, TNF neutralization also reduced renal *Fgf23* expression. TNF may regulate renal *Fgf23* expression through NFκB stimulating orphan nuclear receptor *Nurr1* gene expression [38]. Nurr1 mediates the PTH dependent regulation of *Fgf23* in bone [37]. *Nurr1* was upregulated in PKD kidneys and predominantly localized in the cell nucleus whereas in wild type kidneys it was localized in the cytoplasm. Nurr1 nuclear localization often overlapped with renal FGF23 protein expression. Thus, Nurr1 may contribute to renal FGF23 expression.

In patients with CKD, TNF increases with ascending FGF23 quartiles and correlates with FGF23 levels independent of renal function and measures of mineral metabolism [14]. Likewise, markers of inflammation correlate with ascending FGF23 quartiles in non-CKD stroke patients [16]. Inoculation of mice with LPS or bacteria stimulates serum FGF23 levels [23, 27]. In the diabetic nephropathy rat model, renal FGF23 was reduced by ramipril, an angiotensin-converting enzyme inhibitor [56], which also reduces inflammation [59]. Non-renal diseases characterized by inflammation such as inflammatory bowel disease (IBD) or osteoarthritis are linked to elevated plasma FGF23 [17, 18]. Patients with IBD or mouse

colitis models show elevated FGF23 levels, lower 1,25-((OH)$_2$ vitamin D$_3$$_2$D and impaired intestinal phosphate absorption [17, 60-63]**Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.**. These disturbances are partially caused by TNF and in patients with IBD, TNF neutralizing therapy can reverse some of these abnormalities. We tested whether inflammation *per se* without renal disease could increase FGF23. Consistently, in *Il-10* KO mice, a model of IBD, plasma FGF23 increased and was reduced by TNF neutralization without affecting renal function parameters. Thus, extrarenal inflammation also stimulates FGF23 levels in mouse models and may play a role in humans.

David et al. reported that 6 hours after administration of the inflammatory cytokine IL-1β only cFGF23 increased while it required 4 days of consecutive IL-1β injections to increase also iFGF23 levels [24], whereas Onal et al showed higher FGF23 levels already 3 hrshours after IL-1β injection [27].[27]. We demonstrate that TNF administration in wild type mice stimulated plasma iFGF23 levels within 2448 hours without altering plasma phosphate, and creatinine, or urea levels but increasing fractional excretion of phosphate demonstrating that FGF23iFGF23 is differently regulated by inflammation and phosphatefunctional. TNF may exert even faster effects as indicated by higher FGF23 levels in mice 3 hours after TNF injection [27]. The stimulation of *Fgf23* mRNA expression by TNF was confirmed *in vitro* in primary mouse osteocytes and comparable to the effect of 1,25-((OH)$_2$ vitamin D$_3$$_2$D. TNF but not 1,25-((OH)$_2$ vitamin D$_3$$_2$D increased *Nurr1* and *Galnt3* expression in primary osteocytes suggesting that TNF but not 1,25-((OH)$_2$ vitamin D$_3$$_2$D may regulate *Fgf23* expression in a Nurr1-dependent manner. TNF may also modulate FGF23 protein stability by regulating the expression of *Galnt3* which mediates the O-glycosylation of FGF23 making it more resistant to proteolytic degradation [43]. In bone, C-terminal DMP-1 binds to PHEX and thereby inhibits *Fgf23* expression [64]. In primary osteocytes, *Dmp1* expression was strongly decreased by TNF and 1,25-((OH)$_2$ vitamin D$_3$$_2$D. The upregulation of *Fgf23* expression by TNF and 1,25-((OH)$_2$ vitamin D$_3$$_2$D is paralleled by the downregulation of its suppressor. Our data expand previous

observations in IDG-SW3 mouse osteocyte cells where TNF, IL-1β, and LPS increased *Fgf23* and reduced *Dmp1* mRNA expression [22]. TNF also stimulated *Fgf23* mRNA expression in rat UMR106 osteosarcoma cells and is required to increase circulating FGF23 levels in a mouse obesity model [25]. Deletion of an 16kb enhancer element in the *Fgf23* murine gene abolishes TNF induced FGF23 increases and reduces the effect of LPS and IL-1β on circulating FGF23 levels without altering bone structure or plasma phosphate and PTH. Induction of *Fgf23* mRNA in various organs is organ-specifically responsive to LPS, TNF and IL-1β and the deletion of the enhancer suggesting a complex and cell- and/or organ-specific regulation [27]. The enhancer element is also required for the early induction of FGF23 in the oxalate nephropathy model [27]. Thus, our work demonstrates the critical role of TNF in inducing FGF23 production and thereby complements previous work that identified a genetic element responding to TNF and possibly ~~some~~ other regulators of *Fgf23* mRNA transcription [27]. Furthermore, we expand these observations from kidney disease to at least one other clinically important condition, inflammatory bowel disease.

IL-6 has been recently identified as another important proinflammatory cytokine that associates with FGF23 levels in the CRIC cohort [14] and that stimulates *Fgf23* mRNA in the IDG-SW3 osteocyte cell line [22]. Durlacher-Betzer et al. showed increased expression of IL-6 in kidney of folic-acid and adenine AKI and CKD mouse models and a partly blunted increase of circulating FGF23 levels in Il-6 deficient mice treated with adenine [28]. While IL-6 may participate in the regulation of FGF23 in CKD, IL-6 plays also an important role in normal bone biology and IL-6 deficient mice have altered bone architecture [65, 66]. Thus, IL-6 may contribute to the upregulation of FGF23 in early CKD but TNF may act either upstream or is a critical permissive factor as indicated by the complete normalization of FGF23 levels in our experiments. In our population-based cohort, TNF but not IL-6 associated with intact FGF23 levels further strengthening the concept that TNF may play a central role in mediating effects of inflammation on bone.

Renal hypoxia is a common complication in CKD kidneys [44, 45]. Hypoxia increased *Fgf23* expression in UMR-106 rat osteosarcoma cells, and plasma cFGF23 but not iFGF23 in rats under hypobaric hypoxia conditions [21]. We cultured MC3T3-E1 cells, a mouse preosteoblast cell line and primary mouse osteoblasts for 24 and 48 hours in 0.2% hypoxia and we did not observe any stimulation of *Fgf23* expression. In contrast, hypoxia suppressed the stimulatory effect of 1,25-~~((~~(OH)$_2$~~ vitamin D$_3$~~$_2$D on *Fgf23*. TNF and IL-1β increase HIF-1 binding to DNA under normoxia while in combination with hypoxia both cytokines strongly increase HIF-1 activity [67]. IL-1β but not TNF enhance nuclear accumulation of HIF-1α in a hepatoma cell line [67] and increase FGF23 mRNA expression in bones and kidneys [24]. Inhibition of HIF-1α attenuated the positive effect of IL-1β on FGF23 expression [24]. Combined with the fact that we did not find any effect of constitutively activated HIF-1α in *Vhl* KO animals as well as in primary kidney cells lacking *Vhl*, these results suggest that the HIF-1α mediated upregulation of *Fgf23* expression may depend on IL-1β or other factors such as erythropoietin [11, 24, 68, 69].

In summary, TNF stimulates ~~FGF23~~iFGF23 in renal and non-renal inflammatory mouse models and in primary bone cell culture; triggers renal *Fgf23* expression in CKD animal models and is positively associated with plasma iFGF23 in a population-based cohort~~; it triggers renal *Fgf23* expression in CKD animal models.~~. These findings question the concept that the early rise in plasma FGF23 in CKD is ~~a specific marker of the severity of~~solely to balance plasma phosphate while kidney ~~disease as~~function declines. The data suggest ~~or if~~ that other non-renal inflammatory processes may strongly impact on plasma FGF23 levels. Our study~~ also provides new insights into possible pathological mechanisms through which inflammatory diseases may be linked to bone health. It also~~ suggests novel therapeutic options to reduce excessive FGF23 levels in kidney and other diseases as drugs lowering TNF are widely clinically used and have proven to be safe in humans.

# Methods

## SKIPOGH cohort

We obtained 1098 out of 1131 human EDTA plasma samples from SKIPOGH cohort (Swiss Kidney Project on Genes in Hypertension) [29, 70, 71]. Plasma iFGF23 was measured with the human intact FGF23 ELISA kit (Immutopics International, USA). For statistical modeling the following 18 previously determined parameters were used: plasma calcium, phosphate, ferritin, transferrin, iron, 1,25(OH)$_2$ ~~vitamin D$_3$~~$_2$D, 25(OH) vitamin $D_3$, PTH, TNF, IFNγ, IL-1β, IL-6, IL-10 and cFGF23 as well as body mass index, age, sex and estimated renal function calculated by the CKD-EPI equation.

Exclusion criteria followed the pipeline described in the Table S3. Participants with incomplete data sets (n = 261) were excluded. TNF followed a bimodal distribution with 40 values close to undetectable (TNF < 1 pg/ml) without continuity with the rest of the distribution, highly suggestive for measurement failures. Therefore the 40 participants with TNF < 1 pg/ml were excluded from the study. Next, the ratio between iFGF23 (detects only iFGF23) and cFGF23 (detects iFGF23 and cFGF23) was calculated. One Ru/ml cFGF23 corresponds to 1.5 pg/ml iFGF23 (information provided by Immutopics); participants with ratios higher than 1.5 were excluded (n = 40). To avoid confounding effects by drug intake we eliminated 4 major drug categories that interact with FGF23 metabolism: 1) calcium, phosphate and magnesium (n = 41); 2) inflammation (pro or anti-inflammatory) (n = 390); 3) iron metabolism (n = 6); 4) kidney function (i.e. diuretics) (n = 54) (Table S4). A total of 361 participants were excluded due to intake of drugs of one or more of these drug categories. The final dataset contains either 429 participants (198 female / 231 male) with or 790 (424 female / 366 male) without drug exclusion criteria.

## Animals

*Pkd1* floxed/floxed (*Pkd1$^{fl/fl}$*) tamoxifen inducible *cre* mice were kindly provided by Gregory Germino [33, 72]. *Cre* recombinase expression is under the control of the β-actin promoter which drives high levels of expression in most tissues [33]. Male and female

*Pkd1*$^{fl/fl}$*, cre+* and *Pkd1*$^{fl/fl}$*, cre-* mice were used. Cre recombinase activity was induced at postnatal days 15, 17, and 19 by injecting pups with 100 µl tamoxifen (2.5 mg/ml) in corn oil causing slow onset disease [33]. Without further interventions, 24-hour urine was collected from 6 and 12 weeks old animals (e.g. 3 or 9 weeks after induction, respectively) which were thereafter sacrificed to collect plasma and organs. For TNF blockade, animals were treated at the age of 11-12 weeks with a single i.p. injection of 0.5 mg *InVivo*MAb anti-Tnfα (Clone XT3.11, Lot4653-1/0413, BioXCell, USA) or *InVivo*MAb rat IgG1 (Clone HRPN, Lot 5339/1014, BioXCell, USA) [73, 74]. Twenty-four hours after antibody application, animals were sacrificed and plasma and organs were collected. ~~Unilateral ureteral ligation was performed in 6 weeks old C57/Bl6J mice purchased from Jackson laboratory (Bar Harbor, ME). After 14 days, animals were sacrificed and organs were collected.~~ The effect of TNF in wild-type mice was assessed by injecting ~~12~~13 weeks old C57Bl/6J mice on two consecutive days with 2 µg TNF. After ~~72~~48 hours, plasma and organs were collected.

Nephropathy was induced in 10 to 12 weeks old C57Bl/6J mice. After 3 days of adaptation with calcium-free diet (irradiated S7042-E005S, Sniff Spezialdiäten GmbH, Germany), mice were fed for 10 days with either calcium free diet or 0.67% oxalate in calcium-free diet (irradiated S7042-E010) followed by a 5-day recovery phase in standard diet (3433, Kliba, Kaiseraugst, Switzerland). Forty-eight hours prior to sacrifice, mice received a single i.p. injection of 0.5 mg anti-TNF or isotypic IgG1 control. Mice were sacrificed and plasma and organs were collected.

~~Il-10~~*Il10* deficient mice (~~*Il-10*~~*Il10*$^{-/-}$) develop spontaneous colitis and were used as a non-renal inflammatory disease model [50]. ~~*Il-10*~~*Il10*$^{-/-}$ mice between 12-14 weeks were sacrificed to collect plasma and organs. ~~*Il-10*~~*Il10*$^{-/-}$ mice were treated with a single i.p. injection of 0.5 mg *InVivo*MAb anti-Tnfα (Clone XT3.11, Lot4653-1/0413, BioXCell, USA) or *InVivo*MAb rat IgG1 (Clone HRPN, Lot 5339/1014, BioXCell, USA) [73, 74] 48 hours prior to sacrifice and plasma and organs were collected. For some experiments, kidneys from kidney-specific von-Hippel-Lindau deficient mice were used [46]. All animal studies were

performed according to protocols approved by the legal authority (Veterinary Office of the Canton of Zurich or the Committee on Animal Research, University of California San Francisco).

## Plasma and urine analysis

Blood and 24 hours urine were collected from *Pkd1*$^{fl/fl}$*, cre*+ and *Pkd1*$^{fl/fl}$*, cre*- mice at 6 and 12 weeks after birth. Briefly, *Pkd1*$^{fl/fl}$*, cre* mice were kept for three days in metabolic cages (Tecniplast, Italy) whereas the last day was used for 24 hours urine collection. Afterwards mice were anesthetized with isoflurane and blood was collected from the heart. Plasma and urine aliquots were rapidly frozen and stored at -80 °C until measurement. Urine and plasma laboratory analyses were performed on a UniCel DxC 800 Synchron (Beckman Coulter, Switzerland) by the Zurich Integrative Rodent Physiology (ZIRP) core facility. The ratio of the maximum rate of tubular phosphate reabsorption to the glomerular filtration rate (TmP/GFR) was calculated as follows:

$TmP/GFR\ in\ mmol/L = P_P - [U_P \times P_{crea}/U_{crea}]$[75]. The fractional excretion of phosphate was calculated according to the following equation: $FE_{Pi} = (U_{Pi} \times P_{crea}) = (P_{Pi} \times U_{crea}) \times 100$. where $P_{Pi}$, $U_{Pi}$, $P_{crea}$, and $U_{crea}$ refer to the plasma and urinary concentration of phosphate and creatinine, respectively[75]. The plasma concentration of intact FGF23 (Kainos Laboratories, Japan or Immutopics International, USA), cFGF23 (Immutopics International, USA), intact PTH (Immutopics International, USA) and TNF (Bio-Techne AG, Switzerland) were measured by enzyme-linked immunosorbent assays according to the manufacturers protocols.

## Cell culture

All cell culture reagents were from Life Technologies Europe B.V. (Switzerland) unless stated otherwise. Two to four month old *Pkd1*$^{fl/fl}$*, cre* mice (4-6 mice per experiment, male and female mixed) were sacrificed with carbon dioxide. Tibias and femurs from the hindlegs were harvested. The epiphyses were cut and bones were flushed with Hank's

Balanced Salt Solution (HBSS) containing 1% penicillin streptomycin (Pen Strep) to remove the bone marrow. Bones were cut into small pieces of 1-2 $mm^2$. Bone cell extraction was performed according to established protocols [39, 40]. Briefly, small bone pieces were repeatedly digested with either a solution containing 2 mg/ml collagenase type II, 0.05% (w/v) soybean trypsin inhibitor (Sigma-Aldrich, Switzerland), 20 mM HEPES, 1% Pen Strep in HBSS or 10 nM EDTA, 1% fetal bovine serum (FBS), 1% Pen Strep in phosphate buffered saline (PBS) for 25 min at 37°C. Cells from digestion steps 6-9 or cells and bone pieces from digestion step >9 were cultured for 2 weeks in an osteogenic medium (minimal essential medium α (memα) containing 10% FBS, 1% Pen Strep, 50 µg/ml 2-phospho-L-ascorbic acid trisodium salt (Sigma-Aldrich, Switzerland), and 1 mM β-glycerophosphate (Sigma-Aldrich, Switzerland)). After 2 weeks, cells were supplemented for 24 hours with either 10 nM 1,25(OH)~~)~~$_2$~~ vitamin D~~$_3$~~)$_2$D (CaymanChemical, USA) or 10 ng/ml mouse TNF (R&D Systems, USA) and total mRNA was extracted.

MC3T3-E1 subclone 4 preostoblast cells (CRL-2593, Lot 59899932, ATCC France) passage 17/4 were expanded for 4-5 days with MEMα medium supplemented with 10% FBS and 1% PenStrep. After reaching 80-90% confluence, MC3T3-E1 cells were trypsinized and plated in collagen coated 6-well plates (80'000 cells/well). Medium was changed to osteogenic differentiation medium (MEMα supplemented with 10% FBS, 1% PenStrep, 50µg/ml 2-phospho-L-ascorbic acid trisodium salt (Sigma-Aldrich, Switzerland), and 1 mM beta glycerophosphate (Sigma-Aldrich, Switzerland)). After 2 weeks differentiation along the osteogenic lineage cells were supplemented for 24 or 48 hours with either 10 nM 1,25(OH)$_2$~~ vitamin D~~$_3$$_2$D (CaymanChemical, USA) or an equal amount of ethanol and incubated for 24 or 48 hours under hypoxic (0.2% $O_2$) or normoxic conditions. Hypoxia experiments were performed in a gas-controlled workstation (InvivoO$_2$, Baker Ruskinn, UK).

## RNA extraction, reverse transcription and qPCR

Organs and scraped colonic mucosa were harvested and rapidly frozen in liquid nitrogen. Tissues were homogenized using either a Precellys homogenizer or a liquid nitrogen cooled mortar and pestle (bone). Total mRNA from bone as well as from cultured cells was extracted with TRIzol (Life Technologies Europe B.V., Switzerland) followed by purification with RNeasy Mini Kit (Qiagen, Switzerland) according to the manufacturers protocol. Total mRNA from kidney and colonic mucosa were extracted with RNeasy Mini Kit (Qiagen, Switzerland) according to the ~~manufacturers~~manufacturer's protocol. DNAse digestion was performed using the RNase-free DNAase Set (Qiagen, Switzerland). Total RNA extractions were analyzed for purity and concentration using the NanoDrop ND-1000 spectrophotometer (Wilmington, Germany). RNA samples were diluted to a final concentration of 100 ng/µl and cDNA was prepared using the TaqMan Reverse Transcriptase Reagent Kit (Applied Biosystems, Roche, Foster City, CA). In brief, in a reaction volume of 40 µl, 300 ng of RNA was used as template and mixed with the following final concentrations of RT buffer (1x): $MgCl_2$ (5.5 mmol/l), random hexamers (2.5 µmol/l), dNTP mix (500 µmol/l each), RNase inhibitor (0.4 U/µl), multiscribe reverse transcriptase (1.25 U/µl), and RNAse-free water. Reverse transcription was performed with temperature conditions set at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min on a thermocycler (Biometra, Germany). Quantitative PCR (qPCR) was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers for genes of interest were designed using Primer 3 software. Primers were chosen to span exon - exon boundaries to exclude the amplification of contaminating genomic DNA (primer and probe sequence see Table S5). The specificity of all primers was tested and always resulted in a single product of the expected size (data not shown). Probes were labeled with the reporter dye FAM at the 5'-end and the quencher dye TAMRA at the 3'-end (Microsynth, Switzerland). qPCR reactions were performed using the KAPA PROBE FAST qPCR Kit (KappaBiosystems, USA) or PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Switzerland).

## Protein extraction and Western blot analysis

Organs were rapidly frozen in liquid nitrogen. Tissues were homogenized in homogenization buffer containing 0.27 M sucrose, 2 mM EDTA (pH8), 0.5% NP-40, 60 mM KCl, 15 mM NaCl, 15 mM HEPES (pH7.5) (all Sigma-Aldrich, Switzerland) and complete protease inhibitor cocktail (Roche, Switzerland) using Precellys homogenizer. Nuclei were separated by a sucrose cushion and resuspended in a nuclear extraction buffer containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA (pH 8), 1 mM DTT and 1 mM PMSF (all Sigma-Aldrich, Switzerland). BBM vehicles were prepared using the $Mg^{2+}$ precipitation technique [76]. After measurement of protein concentration (Bio-Rad, Hercules, CA, USA), 60 µg of nuclear proteins or 20 ug of BBM proteins were solubilized in loading buffer containing DTT and separated on a 10% polyacrylamide gel. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline/0.1% Tween-20 or 5% bovine serum albumin (BSA) in Tris-buffered saline/0.1% Tween-20 for 60 min, blots were incubated with the primary antibodies: mouse monoclonal anti-phospho-NFκB p65 (Ser536)(7F1) (Cell Signaling Technology, USA; 1:1000) ~~or,~~ rabbit monoclonal NFκB p65 (D14E12) (Cell Signaling Technology, USA; 1:1000), rabbit polyclonal anti-NaPi-IIa ([77]~~homemade~~; 1:3000) or mouse monoclonal anti-β-actin either for 2 h at room temperature or overnight at 4 °C. Membranes were then incubated for 1 h at room temperature with secondary goat anti-rabbit or donkey anti-mouse antibodies (1:5000) linked to alkaline phosphatase (Promega, USA) or HRP (Amersham, MA, USA or R&D Systems, USA). The protein signal was detected with the appropriate substrates using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analyzed using the software Advanced Image Data Analyser AIDA, Raytest to calculate the ratio between phosphorylated protein to total protein.

## Immunofluorescence staining

Mouse kidneys were perfused through the left heart ventricle with a fixative solution containing 3% paraformaldehyde in phosphate buffered saline (PBS). Kidneys were embedded in TissueTec and frozen in liquid nitrogen. Five µm cryosections were cut. Slides were rehydrated with PBS, treated for 5 min with 0.5% SDS in PBS followed by 10 min treatment with 0.5% Triton-X-100 in PBS (Sigma-Aldrich, Switzerland). Unspecific sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Primary antibodies were diluted in 1% BSA in PBS (rat anti-FGF23 clone #283507 (R&D Systems, USA) 1:1000; rabbit anti-Nurr1 N-20 sc-991 (Santa-Cruz, USA) 1:200) and kidney sections were incubated with the primary antibody overnight at 4 °C. After washing with PBS, sections were incubated with the corresponding secondary antibody (1:500) (anti-rabbit DyLight 594 (Jackson ImmunoResearch, Europe), anti-rat NL493 (R&D Systems, USA)), and DAPI (Life Technologies Europe B.V., Switzerland, 1:1000) for 1 h at room temperature. Slides were washed twice with PBS before they were mounted with Dako glycergel mounting medium (Dako, Switzerland). Sections were visualized on a Leica DM 5500B fluorescence microscope and images processed with ImageJ.

## Statistical analysis

Statistics were performed using unpaired Student's t-test, ANOVA, or Two-Way-ANOVA (GraphPad Prism version 7, GraphPad, San Diego, CA) and R programming environment including the nlme, ~~lme4,~~ visreg, ~~splines, rms~~data.table, car, ~~and~~ lmtest, and forestplot packages**Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.**. $P < 0.05$ was considered significant.

The identification of predictors for iFGF23 variation in the SKIPOGH population was performed using linear mixed models with random intercept. The distribution of all parameters was analyzed in histograms. Due to a heavily skewed distribution, IL-6, IL-

10, IFNγ and IL1-β were log-transformed. All parameters were centralized and then normalized by their standard deviations. Linear or nonlinear relationship of each variable with iFGF23 was assessed using a component residual plot. ~~A restricted cubic spline function with 4 knots was applied to 25 (OH) vitamin $D_3$ and eGFR (CKD-EPI).~~However, all parameters were considered linear. Assumptions on the within-group error were checked with plots of the standardized residuals versus fitted values and a Q-Q plot of the residuals. The assumptions on the random effects were checked with a Q-Q plot of the random effects. ~~The Wald test statistic was used to identify the predictors of iFGF23 in the linear mixed model.~~

## Author contributions

Conceptualization, D. E-S., P.H.I.S., and C.A.W; Methodology, D. E-S., P.H.I.S., and C.A.W; Formal analysis, D. E-S. and P.H.I.S.; Investigation, D. E-S., P.H.I.S., B.G., N.G., C.B., M.Z., D.S., M.R., D.A., B.P., M.P., A.L., V.B., and G.-M. F; Resources C.A.W., D.H., F.K., I.F-W., G.R., M. B., F.P., M.F., F.L., R.H.W., S.H.~~B.-~~ and I.F.; Writing – Original Draft, D. E-S. Writing -Review & Editing, D. E-S., P.H.I.S., and C.A.W; Visualization, D. E-S. and P.H.I.S.; Supervision, C.A.W.; Funding Acquisition, C.A.W, all authors read, edited and approved the manuscript.

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## Conflict of interests

C.A. Wagner has been a member of an advisory board to Bayer Pharma AG, and provided consultancy to Medice. No other financial interests are reported.

## Figure legends

### Figure 1

~~TNF is a predictor of plasma iFGF23 in a human cohort.~~**Identification of plasma iFGF23 predictors in a human cohort.** (a) Forest plot showing the fixed effects calculated for all predictors used in the mixed linear model for the subpopulation of 429 participants after all the exclusion criteria applied. Fixed effect estimates (β), standard error, ratio between the estimates and their standard errors (t-value), and associated p-value from a t-distribution. The parameters are ordered by fixed effect estimates. (b) Association between plasma TNF and iFGF23 in the SKIPOGH cohort in a subpopulation of 429 participants after all the exclusion criteria applied. The regression line and confidence band were obtained from the linear mixed model containing all the predictors.

### Figure 2

**FGF23 and inflammation in ~~the kidney of~~ *Pkd1* KO mice.** Plasma FGF23 (a) and TmP/GFR (b) as well as renal *Tnf* (a~~e~~c) and renal *Tgfb* (~~b~~fd) mRNA expression relative to 18SrRNA in *Pkd1*$^{fl/fl}$*, cre-* (white squares) and *Pkd1*$^{fl/fl}$*, cre+* (black squares) animals after 6 and 12 weeks. Phosphorylation of NFκB p65 (~~c~~ge) in the nuclear fraction of total kidney protein homogenates in *Pkd1*$^{fl/fl}$*, cre-* (white squares) and *Pkd1*$^{fl/fl}$*, cre+* (black squares) animals after 12 weeks. Renal (f) and bone (g) *Fgf23* mRNA expression relative to 18SrRNA in *Pkd1*$^{fl/fl}$*, cre-* (white squares) and *Pkd1*$^{fl/fl}$*, cre+* (black squares) animals after 12 weeks. ND = not detected. Two-way ANOVA with Bonferroni correction (a~~,b~~ - d) or unpaired t-test (~~c~~e - g), * p<0.05.

### Figure 3

**TNF neutralization lowers FGF23 in *Pkd1* KO mice.** Plasma TNF (a), iFGF23 (b), phosphate (c), and ~~plasma~~ urea (d) levels, bone (e) and renal (f) *Fgf23* ~~(e), renal *Fgf23* (f),~~, renal *Tnf* (g), and renal *Tgfb* (h) mRNA expression relative to *Hprt*~~,~~ as well as abundance of NaPi-IIa (i) in~~a~~t the renal BBM relative to β-actin 24 hours after injection of 0.5mg isotypic IgG control or anti-TNF neutralizing antibodies in 11-12 weeks old *Pkd1*$^{fl/fl}$*, cre-* (white squares) and *Pkd1*$^{fl/fl}$*, cre+* (black squares) animals. ND = not ~~detectable~~detected. Two-way ANOVA with Bonferroni correction * p<0.05.

### Figure 4

**TNF stimulates FGF23 *in vivo* and *in vitro*.** Plasma iFGF23 (a), TNF (b), phosphate (~~b~~c), creatinine (~~c), and~~d), urea (~~d) levels~~e) and $FE_{Pi}$ (f) as well as bone (g), spleen (h) thymus (i) and bone marrow (j) Fgf23 mRNA expression relative to *Hprt* (g,h) or *18SrRNA* (i,j) 48 hours after two consecutive injections of vehicle or 2 µg recombinant mouse TNF in 12 weeks old wild type mice. Unpaired t-test * p<0.05. Fold increase of *Fgf23* (~~e~~k), *Dmp1* (~~f~~l), *Galnt3* (~~g~~m), and *Nurr1* (~~h~~n) mRNA expression compared to untreated control in primary murine osteocytes after stimulation with 1,25(OH)$_2$~~ vitamin D$_3$~~2D (white squares) or 10ng/ml TNF (black squares) for 24 hours. Single experiments were normalized to their untreated control (dashed line = 1). Number of independent experiments 9-10; One-way ANOVA with Bonferroni correction * p<0.05 compared to 1,25(OH)$_2$~~ vitamin D$_3$~~2D treated cells, # p<0.05 compared to untreated cells.

**Figure 5**

**TNF neutralization lowers ~~also~~ plasma iFGF23 in mice with oxalate nephropathy.** Oxalate-nephropathy was induced in wild type mice. Plasma iFGF23 (a), plasma TNF (b), renal *Tnf* (c) mRNA expression relative to *Hprt*, plasma phosphate (d), urinary phosphate to creatinine ratio (e), plasma creatinine (~~e),~~f) and plasma urea (~~f~~g) 48 hours after injection of 0.5 mg isotypic IgG control or anti-TNF neutralizing antibodies in control diet (white squares) and oxalate nephropathy (black squares) induced mice. One-way ANOVA with Bonferroni correction * $p<0.05$.

**Figure 6**

**Colonic inflammation increases plasma iFGF23 via TNF in *Il-10* KO mice.** Plasma iFGF23 (a) levels and colonic *Tnf* (b) mRNA expression relative to 18SrRNA in 14 weeks old *Il-10*$^{+/+}$ and *Il-10*$^{-/-}$ mice. Plasma iFGF23 (c), phosphate (d), creatinine (e), and urea (f) levels as well as abundance of NaPi-IIa at the renal BBM 48 hours after injection of 0.5 mg isotypic IgG control or anti-TNF neutralizing antibodies in 12 weeks old *Il-10*$^{-/-}$ mice. Unpaired t-test * $p<0.05$.

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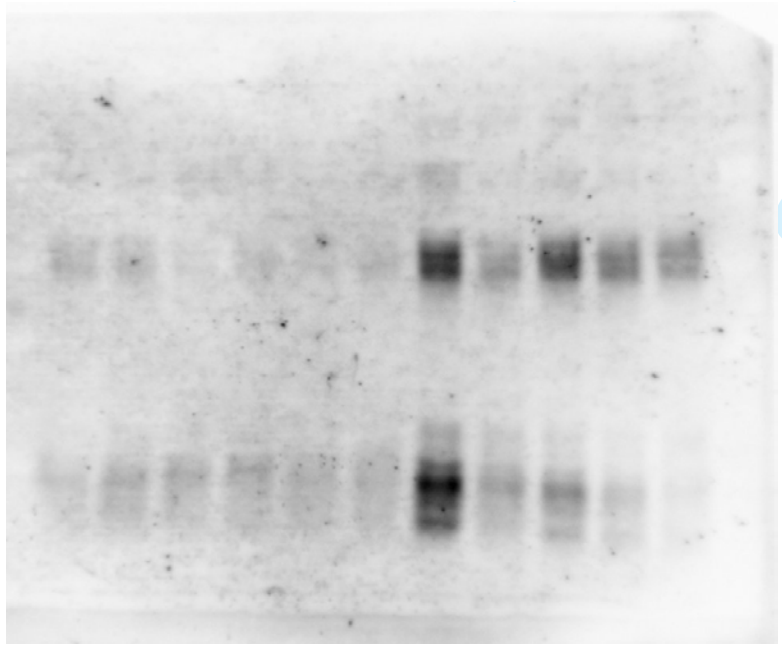
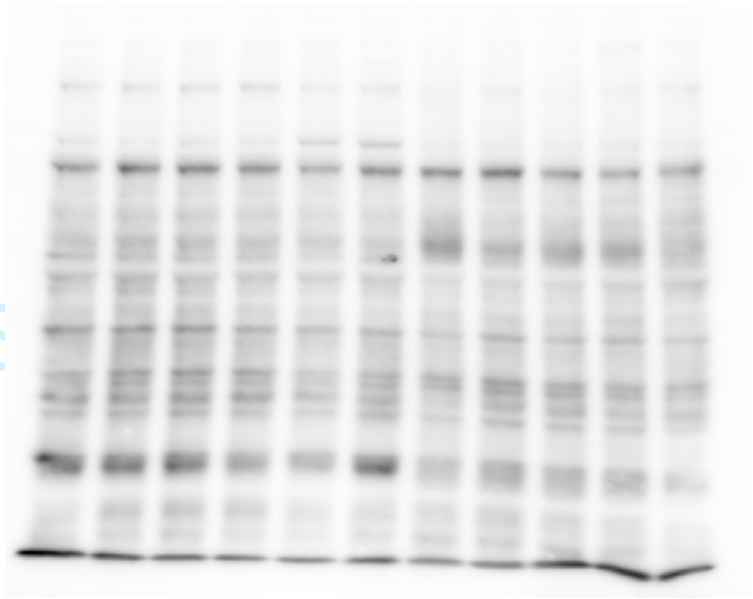
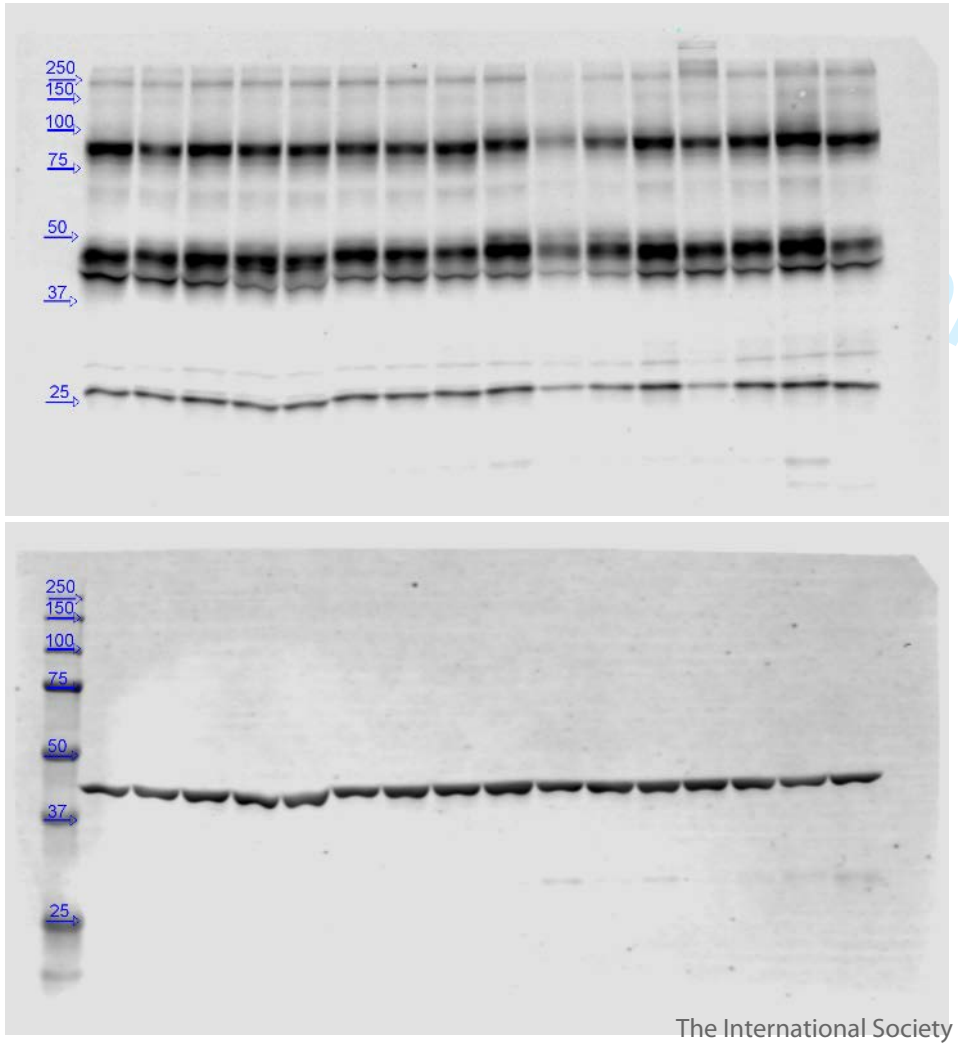
# Figure 2





# Figure 3



# Figure 6