



Fibroblast Growth Factor 23 Expression Is Increased in Multiple Organs in Mice With Folic Acid-Induced Acute Kidney Injury

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Fibroblast growth factor 23 (FGF23) regulates phosphate homeostasis and vitamin D metabolism. In patients with acute kidney injury (AKI), FGF23 levels rise rapidly after onset of AKI and are associated with AKI progression and increased mortality. In mouse models of AKI, excessive rise in FGF23 levels is accompanied by a moderate increase in FGF23 expression in bone. We examined the folic acid-induced AKI (FA-AKI) mouse model to determine whether other organs contribute to the increase in plasma FGF23 and assessed the vitamin D axis as a possible trigger for increased *Fgf23* gene expression. Twenty-four hours after initiation of FA-AKI, plasma intact FGF23 and 1,25(OH)₂D were increased and kidney function declined. FA-treated mice developed renal inflammation as shown by increased *Tnf* and *Tgfb* mRNA expression. *Fgf23* mRNA expression was 5- to 15-fold upregulated in thymus, spleen and heart of FA-treated mice, respectively, but only 2-fold in bone. Ectopic renal *Fgf23* mRNA expression was also detected in FA-AKI mice. Plasma FGF23 and *Fgf23* mRNA expression in thymus, spleen, heart, and bone strongly correlated with renal *Tnf* mRNA expression. Furthermore, *Vdr* mRNA expression was upregulated in spleen, thymus and heart and strongly correlated with *Fgf23* mRNA expression in the same organ. In conclusion, the rapid rise in plasma FGF23 in FA-AKI mice is accompanied by increased *Fgf23* mRNA expression in multiple organs and increased *Vdr* expression in extra osseous tissues together with increased plasma 1,25(OH)₂D and inflammation may trigger the rise in FGF23 in FA-AKI.

Keywords: fibroblast growth factor 23, acute kidney injury, 1,25(OH)₂D, inflammation, vitamin D receptor, tumor necrosis factor

INTRODUCTION

Fibroblast growth factor 23 (FGF23) is a potent regulator of phosphate homeostasis and vitamin D metabolism (Shimada et al., 2004). FGF23 binds to its co-receptor α Klotho and FGFR receptors 1c, 3 and 4 to activate mitogen activated protein kinase (MAPK) signaling pathway (Kurosu et al., 2006; Ranch et al., 2011; Chen et al., 2018). In the kidney, FGF23 inhibits phosphate reabsorption and suppresses 1,25-dihydroxyvitamin D (1,25(OH)₂D) production by downregulation of *Cyp27b1* and upregulation of *Cyp24a1* gene expression (Shimada et al., 2004; Perwad et al., 2007). *Cyp27b1* and *Cyp24a1* encode the enzymes 1 α -hydroxylase and 24-hydroxylase, respectively, which are responsible for 1,25(OH)₂D

synthesis (Takeyama et al., 1997) and degradation (Ohyama et al., 1991). FGF23 is mainly expressed in bone but low levels of expression are detected in thymus, spleen and heart (Fon Tacer et al., 2010).

In patients with acute kidney injury (AKI), plasma and urinary C-term FGF23 (cFGF23) levels rise rapidly after onset of AKI and are independently associated with AKI progression and adverse outcomes (Leaf et al., 2013, 2016, 2017). Increase in cFGF23 levels is independent of changes in plasma PTH, phosphate and vitamin D metabolites (Leaf et al., 2016). In pediatric and adult patients with AKI following cardiac surgery, there is a transient increase in plasma intact FGF23 (iFGF23) levels which subsequently normalize (Hanudel et al., 2016; Leaf et al., 2016). Preoperative plasma cFGF23 levels in pediatric and adult patients undergoing cardiac surgery are associated with increased risk of AKI and post-operative mortality (Speer et al., 2015; Hanudel et al., 2016). Although, pre- and post-operative cFGF23 levels serve as prognostic tools to predict post-operative AKI severity and complications (Speer et al., 2015; Leaf et al., 2016), the mechanisms by which FGF23 levels increase in AKI are poorly understood.

In the folic acid-induced AKI (FA-AKI) mouse model, plasma FGF23 levels rise rapidly after onset of AKI and are partially independent of phosphate and 1,25(OH)₂D suggesting that there are other contributing mechanisms (Christov et al., 2013). FGF23 production is elevated in bone of FA-AKI mice (Christov et al., 2013) and systemic FGF receptor blockade reduces bone *Fgf23* mRNA expression and normalizes FGF23 levels (Hassan et al., 2016). Considering that FGF23 production in bone is only modestly increased in FA-AKI, other organs may contribute to the massive increase in FGF23 levels (Christov et al., 2013; Hassan et al., 2016). In hemorrhagic shock and sepsis-induced AKI rat model, *Fgf23* gene expression is increased in bone marrow and is erythropoietin (EPO)-dependent (Toro et al., 2018).

In this study, we determined whether other organs contribute to the increase in plasma FGF23 levels in the FA-AKI mouse model and assessed the vitamin D axis as possible mechanism for stimulation of FGF23 production in multiple organs.

METHODS

Animals

Eight-week-old male and female C57Bl/6J mice from in house breeding were used for all experiments. Animals were allowed *ad libitum* access to water and food (PicoLab Mouse Diet 20 5058). AKI was induced by intraperitoneal injection of folic acid (250 mg/kg; F7876, Sigma-Aldrich) or vehicle (0.15 M NaHCO₃) as previously described (Christov et al., 2013). After 24 h, mice were anesthetized with Ketamine/Xylazine before blood and organs were collected. All animal studies were performed according to approved protocols (Institutional animal care and use committee, San Francisco).

Plasma Analysis

Blood was drawn from the heart and collected in BD Microtainer™ Tubes containing Lithium Heparin (Becton, Dickinson and Company) for plasma separation. Plasma was aliquoted, rapidly frozen and stored at -80°C. Plasma phosphate, creatinine and BUN were measured with Phosphorus Liqui-UV® test, Creatinine LiquiColor® test, or Urea Nitrogen (BUN) Liqui-UV® (Rate), respectively (EKF Stanbio, United States). The plasma concentration of iFGF23 (Immutopics International, United States) and 1,25(OH)₂D (Immunodiagnostic Systems Inc, United Kingdom) were measured by enzyme-linked immunosorbent assays according to manufacturers' protocols.

RNA Extraction, Reverse Transcription, and qPCR

Organs were harvested and rapidly frozen in liquid nitrogen. Tissues were homogenized using either a BeadBug™ microtube homogenizer or a liquid nitrogen cooled mortar and pestle. Total RNA from bone (one femur and tibia each), bone marrow (spin down from femur and tibia), heart, spleen, and thymus was extracted with TRIzol (Life Technologies Europe B.V., Switzerland) and total RNA from kidney with NucleoSpin® RNA lysis buffer followed by purification with NucleoSpin® RNA Miniprep (Clontech, United States) according to the manufacturers' protocol including Dnase1 digestion. Total RNA extractions were analyzed for purity and concentration using the NanoDrop ND-1000 spectrophotometer. RNA samples were diluted to a final concentration of 100 ng/μl and cDNA was prepared with reagents from Invitrogen (United States) if not stated otherwise. 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 (1×): MgCl₂ (5.5 mmol/l), random hexamers (2.5 μmol/l), dNTP mix (500 μmol/l each; Bioline Ltd., United States), 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 (Eppendorf). Quantitative PCR (qPCR) was performed using the ABI PRISM 7900HT Detection System (Applied Biosystems). Primers were designed using Primer 3 software (Untergasser et al., 2012). Mouse primer/probe sequences: *Tnf* Fwd 5'-CAGACCCTCACACTCAGATCATCT-3', Rev 5'-CCTCCACTTGGTGGTTTGCT-3', Probe 5'-ATTCG AGTGACAAGCCTGTAGCCCACGT-3'; *Tgfb1* Fwd 5'-CCGC AACAAGCCATCTATG-3', Rev 5'-TGCTTCCCGAATGTCTG ACG-3'; *Klotho* Fwd 5'-CAGCTCCAGGCTCGGGTA-3', Rev 5'-AGGTGTTGTAGAGATGCCAGACTTT-3', Probe 5'-TTG CCCACAACCTACTTTGGCTCATG-3'; *Vdr* Fwd 5'-CACA AGACCTACGACCCAC-3', Rev 5'-CCGGTTCATCATGTC CAGT-3'; *Gus* Fwd 5'-CTCATCTGGAATTTCCGCGA-3', Rev 5'-GGCGAGTGAAGATCCCCTTC-3', Probe 5'-CGAACCAGT CACCGCTGAGAGTAATCG-3'; *Fgf23* Fwd 5'-GACCAGCT ATCACCTACAGATCCA-3', Rev 5'-CGGCGTCTCTGATG

TAATCA-3', Probe 5'-CCCATCAGACCATCTACAGTGCCC TGA-3'; *Cyp27b1* Fwd 5'-CCTCTGCCGAGACTGGGA-3', Rev 5'-TCCCGAAAAAGGAAGTGGGT-3', Probe 5'-TGTTTGCC TTTGCCAGAGGCAC-3'; *Cyp24a1* Fwd 5'-TACGCTGCTG TCACGGAGC-3', Rev 5'-TCTGGATTCCCGGAGAAGTC-3', Probe 5'-CAGTGGAGACGACCGCAAACAGCTT-3'; Primers and probes were purchased either from Elim Biopharma (United States), IDT (United States), or Applied Biosystems (Eukaryotic 18S rRNA Endogenous Control primer probe set). Probes were labeled with the reporter dye FAM at the 5'-end and the quencher dye TAMRA or BHQ1 at the 3'-end. qPCR reactions were performed using the TaqMan Fast Advanced Master Mix or PowerUp SYBR Green Master Mix (Applied Biosystems, United States).

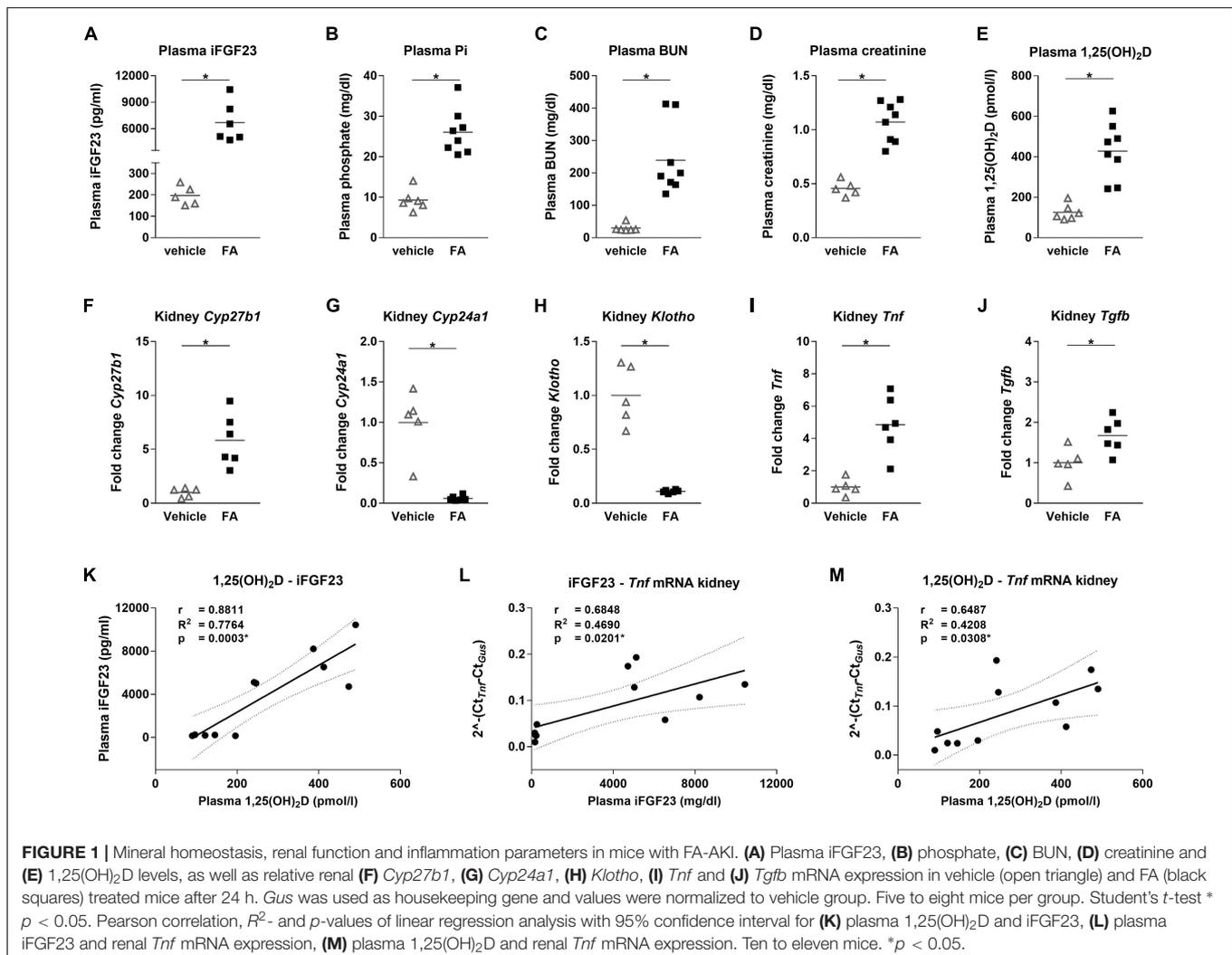
Statistical Analysis

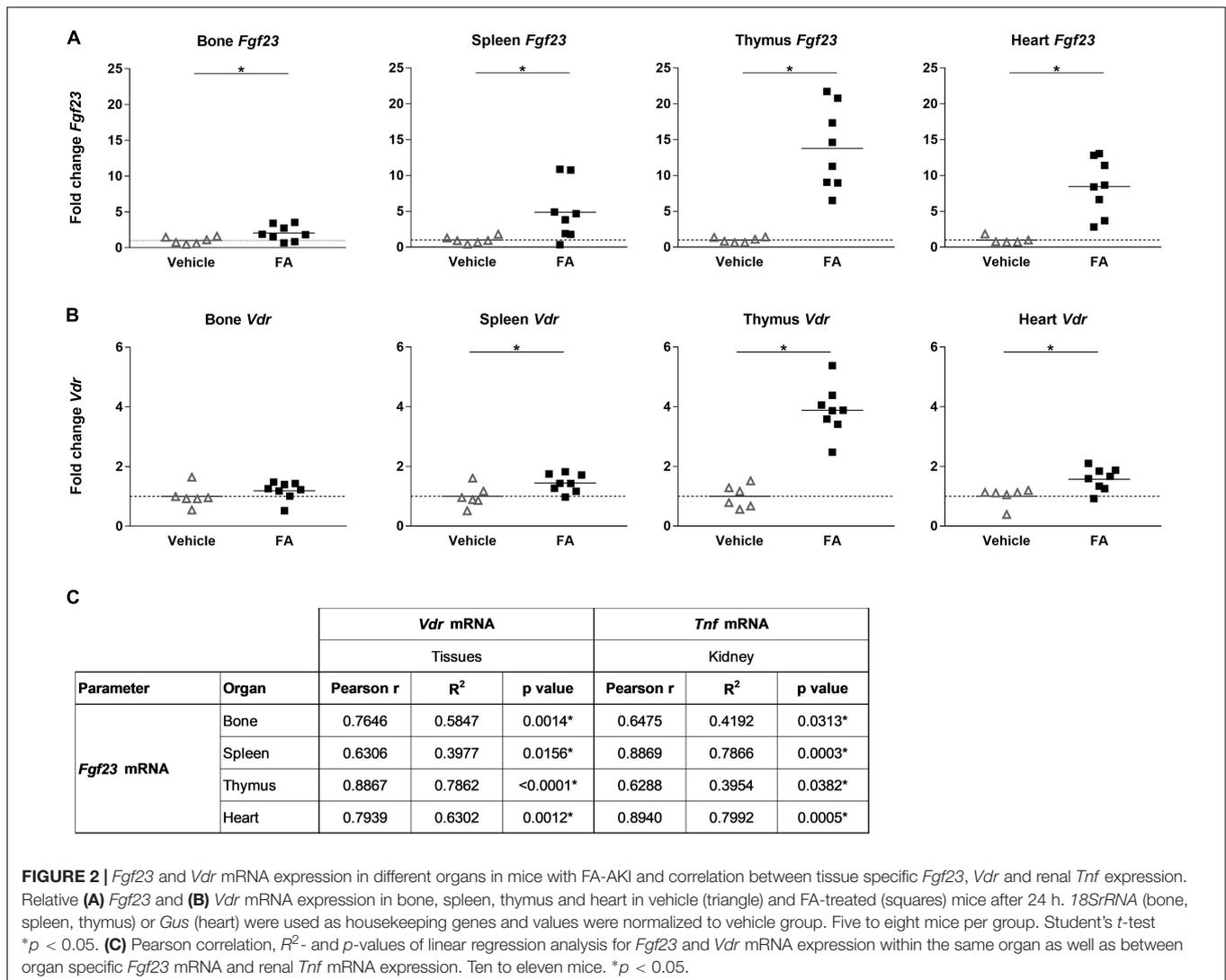
Statistics were performed using unpaired Student's *t*-test and linear regression with Pearson correlation (GraphPad Prism version 7, GraphPad, San Diego, CA, United States). $P < 0.05$ was considered significant.

RESULTS

iFGF23, Vitamin D and Inflammation in FA-AKI

We administered wild type mice with FA or vehicle by intraperitoneal injections to determine the effect of FA-AKI on *Fgf23* gene expression. Twenty-four hours after AKI induction, plasma iFGF23 and phosphate increased significantly while kidney function declined in FA-treated mice (Figures 1A–D). Further, plasma 1,25(OH)₂D increased by 3-fold in FA-treated mice which was accompanied by 6-fold increase in renal *Cyp27b1* and 17-fold reduction in *Cyp24a1* mRNA expression (Figures 1E–G). FA-AKI reduced *Klotho* mRNA expression and triggered renal inflammation as demonstrated by increased *Tnf* and *Tgfb* mRNA expression (Figures 1H–J). We performed linear regression analyses to determine the relationships between plasma iFGF23, 1,25(OH)₂D, and inflammation in FA-AKI. Plasma iFGF23 significantly correlated with plasma 1,25(OH)₂D and both plasma iFGF23 and 1,25(OH)₂D significantly correlated with





renal mRNA expression of the inflammatory cytokine *Tnf* (Figures 1K–M).

Multiple Organs Contribute to Rapidly Increased iFGF23 in FA-AKI Mice

As previously shown, *Fgf23* mRNA expression was upregulated by 2-fold in bone in FA-treated mice compared to the control group (Figure 2A). Furthermore, *Fgf23* mRNA expression increased significantly in spleen, heart and thymus, by 5-, 8-, and 14-fold, respectively. Ectopic renal *Fgf23* mRNA expression was detected in FA-treated mice while it was absent in the control group (not shown). There was no change in *Fgf23* mRNA expression in whole bone marrow with FA treatment (not shown). Previous studies have shown that activation of vitamin D receptor (VDR) by 1,25(OH)₂D upregulates *Fgf23* expression in bone (Liu et al., 2006). We determined the effect of increased plasma 1,25(OH)₂D on *Vdr* mRNA expression in the organs we analyzed *Fgf23* mRNA expression. We observed a 1.5- to 4-fold upregulation of *Vdr* mRNA expression in FA-treated mice

in spleen, heart and thymus but there was no change in gene expression levels in bone (Figure 2B). *Fgf23* mRNA expression in thymus, spleen, heart, and bone significantly correlated with *Vdr* mRNA expression within the same organ as well as with renal *Tnf* mRNA expression (Figure 2C).

DISCUSSION

In patients with AKI, higher FGF23 levels are associated with AKI progression and increased mortality but mechanisms by which circulating FGF23 increase with kidney injury are unknown (Leaf et al., 2013, 2016, 2017). In the FA-AKI mouse model, increased *Fgf23* mRNA in bone was shown to contribute to the increase in plasma FGF23 (Christov et al., 2013). In this study, we demonstrate that increased *Fgf23* mRNA expression in multiple organs including bone accompanies the rapid increase in plasma iFGF23 in FA-AKI mice. Furthermore the rise in plasma iFGF23 is paralleled by increased plasma 1,25(OH)₂D. Interestingly, the

two lymphoid organs, thymus and spleen, show a very high induction of *Fgf23* mRNA expression in mice with FA-AKI. The pattern of *Fgf23* expression in lymphoid organs and its rapid increase following AKI suggest that FGF23 plays an important role in the inflammatory response triggered by FA-AKI including increased *Tnf* expression and elevated plasma IL-6 (Moreno et al., 2011; Wen et al., 2012). We confirmed that FA-AKI increases renal mRNA expression of inflammatory cytokines, *Tnf* and *Tgfb*. Moreover, we demonstrate that plasma iFGF23, 1,25(OH)₂D and *Fgf23* mRNA expression significantly correlated with renal *Tnf* mRNA expression. Inflammatory stimuli such as LPS, IL-1 β , and TNF have been shown to increase *Fgf23* mRNA expression *in vivo* and *in vitro* (Ito et al., 2015; Masuda et al., 2015; David et al., 2016). In mice with LPS-induced intermittent chronic inflammation, *Fgf23* mRNA expression in spleen is increased and splenectomy reduces plasma FGF23 levels (Bansal et al., 2017). Whether these cytokines upregulate *Fgf23* mRNA expression in spleen and thymus in FA-AKI need further evaluation.

Ectopic renal FGF23 expression has been reported in animal models of chronic kidney disease and in mice with unilateral ureter obstruction (Zanchi et al., 2013; Spichtig et al., 2014; Smith et al., 2017). Renal FGF23 expression in uremic rats does not contribute to increased circulatory FGF23 levels but local production may further trigger renal fibrosis in these animals (Mace et al., 2017; Smith et al., 2017). Our findings of renal *Fgf23* mRNA expression in FA-treated mice expands the pathological conditions where ectopic renal FGF23 expression is present. Hemorrhagic shock and sepsis-induced AKI in rats result in EPO-EPO receptor-dependent upregulation of *Fgf23* mRNA levels in the bone marrow (Toro et al., 2018). In our study, we did not detect any changes in *Fgf23* mRNA expression in whole bone marrow of FA-AKI mice. AKI disrupts the crosstalk between kidney and heart and contributes to acute reno-cardiac syndrome (Kingma et al., 2015). The resulting cardiac dysfunction is attributed to changes in kidney function, hemodynamics and inflammatory mediators (Kingma et al., 2015). Further studies are needed to determine whether the inflammatory response caused by FA-AKI is responsible for the increase in *Fgf23* mRNA expression in the heart of FA-AKI mice.

In bone, 1,25(OH)₂D activates VDR and allows VDR binding to VDR response elements in the *Fgf23* promoter region to upregulate *Fgf23* mRNA expression (Liu et al., 2006). Therefore, increase in local VDR expression together with high plasma 1,25(OH)₂D may be responsible for increased *Fgf23* mRNA expression seen in different organs in FA-treated mice. Indeed,

we found elevated plasma 1,25(OH)₂D in FA-AKI mice and increased *Vdr* mRNA expression in spleen, thymus and heart but not in bone. Moreover, *Vdr* mRNA expression strongly correlated with *Fgf23* mRNA expression in the same organ. Therefore, activation of 1,25(OH)₂D-VDR signaling pathway may contribute to increase in plasma iFGF23 in FA-AKI mice.

Increased plasma 1,25(OH)₂D in FA-AKI mice was accompanied by increased renal *Cyp27b1* and decreased *Cyp24a1* mRNA expression. These results suggest that FGF23 is unable to regulate renal vitamin D metabolism in FA-AKI mice which could be partially explained by interrupted FGF23 signaling due to a decrease in renal *Klotho* expression with AKI (Hu et al., 2010). Similar to our study, 3/4 nephrectomized rats also demonstrate increased renal *Cyp27b1* and decreased *Cyp24a1* and *Vdr* mRNA expression (Takemoto et al., 2003). However, in patients with CKD and acute renal inflammation plasma 1,25(OH)₂D is low with increased *CYP27b1*, *CYP24a1*, and *VDR* expression (Zehnder et al., 2008). Renal *CYP27b1* expression in these patients was localized to activated macrophages and epithelial cells (Zehnder et al., 2008). Therefore, humans and rodents differ in the renal expression patterns for genes in the vitamin D metabolic pathway.

In summary, we demonstrate that the rapid rise in plasma iFGF23 in mice with FA-AKI is accompanied by increased *Fgf23* mRNA expression in multiple organs including bone, thymus, spleen, heart and kidney. Furthermore, increased plasma iFGF23 levels are paralleled by increased plasma 1,25(OH)₂D and increased *Vdr* mRNA expression in thymus, spleen, and heart.

AUTHOR CONTRIBUTIONS

DE-S and FP conceived the study, provided the methodology, and wrote, reviewed, and edited the manuscript. DE-S performed the formal analysis and visualization process and wrote the original draft of the manuscript. DE-S and MZ contributed to the investigation process. FP gathered resources and supervised the study and acquired funding. All authors read, edited, and approved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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