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Research Article

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High FGF23 Levels Failed to Predict Cardiac Hypertrophy in Animal Models of Hyperphosphatemia and Chronic Renal Failure

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Abbreviations: CKD, chronic kidney disease; CRS, cardiorenal syndrome; FGF23, fibroblast growth factor 23; FGFR4, fibroblast growth factor receptor 4; HF, heart failure; Nx, nephrectomy.

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Abstract

Increased fibroblast growth factor 23 (FGF23) levels are an independent predictor for adverse cardiac events suggesting a role as a link that drives cardiomyopathic changes in cardiorenal syndrome. The search for the underlying mechanism driving this interaction has led to the hypothesis that FGF23 causes pathogenic changes in the heart. Increased serum FGF23 has been independently shown to cause increased cardiac morbidity, mortality, and hypertrophy by signalling through FGF receptor 4. This mechanistic concept was based on preclinical studies demonstrating inhibition of FGF23 signaling through FGF4, which led to suppression of left ventricular hypertrophy and fibrosis in a 2-week rat 5/6 nephrectomy study and a 12-week (2%) high-phosphate diet mouse model in which FGF23 levels were markedly elevated. In this report, renal dysfunction was observed in the 5/6 nephrectomy model, and FGF23 levels were significantly elevated, whereas no changes in left ventricular hypertrophy were observed at 2 or 4 weeks postnephrectomy. Mice placed on a high-phosphate diet that did not cause significant renal dysfunction resulted in significantly elevated FGF23 but no changes in left ventricular hypertrophy. The in vivo studies reported here, which were performed to recapitulate the observations of FGF23 as a driver of cardiac hypertrophy, did not lend support to the FGF23-driven cardiac remodelling hypothesis.

Key Words: FGF23, FGFR4, cardiac hypertrophy, 5/6 nephrectomy, phosphate diet

Heart failure (HF) and chronic kidney disease (CKD) are major public health problems that affect millions of people worldwide and exact enormous healthcare costs each year [1, 2]. When HF and CKD coexist, it is known as cardiorenal syndrome (CRS), whereby a complex interaction of hemodynamic, neurohumoral, and renal factors

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induce acute or chronic dysfunction in both organs and lead to a poorer prognosis [3-5]. Multiple studies have shown that a majority of patients with HF have a history of CKD and, inversely, patients with CKD having HF [4, 6-9]. There has been much debate around the physiological underpinnings of CRS, with reports involving myocardial changes (hypertrophy, atrial fibrillation), vascular system, anemias, nonsteroidal anti-inflammatory drug use, renin-angiotensin system activation, and oxidative stress as part of this complex pathophysiology [3, 5, 6]. It has been demonstrated in the Candesartan in Heart Failure: Assessment of Reduction in Mortality and Morbidity study that patients with reduced estimated glomerular filtration rate had associated decreased left ventricular ejection fraction, increased risk of cardiovascular death, and unforeseen hospital admission [7]. Importantly, data from the observational ADHERE database, which studied the clinical outcomes of more than 100 000 patients with acute decompensated heart failure, showed that ~50% of patients admitted with a diagnosis of acute decompensated heart failure also exhibited stage III renal dysfunction [10].

The search for the underlying mechanisms driving this comorbid interaction has led to the hypothesis that fibroblast growth factor 23 (FGF23) is involved in pathogenic changes to the heart [11-14]. FGF23 is an endocrine growth factor hormone that regulates circulating phosphorus levels and vitamin D metabolism and has been one of the most intensively studied hormones potentially linking cardiovascular disease and CKD [15]. As kidney function declines in CKD, phosphate excretion is perturbed and FGF23 levels increase as a response to phosphate elevation [16, 17]. Recent studies have shown that increased FGF23 levels are an independent predictor for adverse cardiac events suggesting a role as the "missing link" that drives cardiomyopathy changes in CRS [18-22]. Evidence suggests that the pathogenic mechanism of FGF23 is mediated by activation of the FGF receptor 4 (FGFR4) in the heart [11, 12]. This mechanistic concept was based on preclinical studies demonstrating inhibition of left ventricular hypertrophy and fibrosis by treatment with FGFR4 inhibitors in a 2-week rat 5/6 nephrectomy (Nx) study in which FGF23 levels were elevated [12, 23]. Additional evidence for the role of FGF23-induced FGFR4-mediated left ventricular hypertrophy was reported in a 12-week high-phosphate (2%) diet mouse model in which FGF23 levels were markedly elevated [12]. Furthermore, 48-hour FGF23 treatment of isolated neonatal rat ventricular myocytes induced hypertrophy and increased expression levels of hypertrophic genes α- and β-MHC, ANP, BNP, and MCAD in a dose-dependent fashion [11].

Although preclinical and clinical studies have demonstrated elevated FGF23 as a potent risk factor for increased cardiac morbidity and mortality in patients with CKD, there remains uncertainty surrounding the causal role of FGF23 in cardiovascular disease [24, 25]. For example, it has been reported that cardiac hypertrophy was absent in animal models of X-linked hypophosphatemia and patients with human rickets/osteomalacia and increased FGF23 [26, 27]. In addition, it was demonstrated that FGF23 did not have a role in mouse models of left ventricular pressure overload [28].

In this report, we aimed to corroborate the published findings of a relationship between FGF23 levels and cardiac hypertrophy, 2 previously reported rodent models of increased FGF23 and cardiac hypertrophy were conducted: (1) the rat 5/6 Nx, which has been shown to cause severe renal impairment and (2) the 12-week mouse 2% high-phosphate diet known to alter whole-body phosphate homeostasis without significant renal impairment [12, 23, 29, 30].

Materials and Methods

Animal use statement

All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed.

High-phosphate diet study

Mice (C57BL6), 25 to 27 weeks of age were randomized into 2 diet groups: normal diet (Teklad Global 18% protein rodent diet) and high-phosphate diet (Teklad custom diet, 2% available phosphorus diet; 7001), both from Harlan Laboratories. Each group (n = 10) started with a similar mean and standard deviation for body weight. At the end of the 12 weeks, blood was collected from the vena cava and placed into EDTA microtainer tubes and serum separator tubes. Blood from both the tubes were centrifuged at a maximum speed of 12 000*g* and plasma and serum stored at –80°C for FGF23 assessment. Hearts were rinsed in cold 0.9% NaCl, blotted and weighed, and stored at –80°C. The left tibia was excised by dissecting through the knee and ankle joint of the left rear leg. The length of the tibia was measured (proximal to distal heads of the bone) with calipers.

Rat 5/6 Nx study

Three groups, sham (n = 10 for 2-week time point and n = 5 for 4-week time point), 2-week Nx (n = 10)

and 4-week Nx (n = 10) of male Wistar rats weighing 300 ± 20 g were used. Male Wistar rats were provided by BioLasco Taiwan (under Charles River Laboratories Technology Licensee). Space allocation for 2 animals was 45 × 23 × 21 cm. Animals were housed in animal cages and maintained in a controlled temperature (20-24°C) with 12-hour light/dark cycles for at least 3 days in Eurofins Panlabs Taiwan, Ltd. laboratory before use. Free access to standard laboratory diet (Oriental Yeast Co., Ltd., Japan) and autoclaved tap water were granted. All aspects of this work including housing, experimentation, and animal disposal were performed in general accordance with the "Guide for the Care and Use of Laboratory Animals: Eighth Edition" (National Academies Press, 2011) in an AAALAC-accredited laboratory animal facility. In addition, the animal care and use protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Eurofins Panlabs Taiwan, Ltd. All studies were blinded. Animals were randomized before surgery; surgeon was blinded at time of surgery; and imaging, histology, and all other assays were blinded.

On day 0, the animals were anesthetized with pentobarbital sodium (50 mg/kg, IP, bolus injection in a volume of 5 mL/kg) and subjected to subtotal Nx or sham operation (sham surgery controls). After laparotomy, subtotal Nx was started with right Nx followed by ligation of 2 branches (leaving 1 branch intact) of the left renal artery. Postoperatively, the animals were housed individually under constant temperature (20-24°C) with 12-hour light/dark cycles. The animals had free access to water and standard rat chow.

At the end of 2 and 4 weeks after surgery, blood was collected from the vena cava and placed into EDTA microtainer tubes and serum separator tubes. Blood from both the tubes were centrifuged at a maximum speed of 12 000*g* and plasma and serum stored for FGF23 assessment. Hearts were rinsed in cold 0.9% NaCl, blotted and weighed, and stored at −80°C. The left tibia was excised by dissecting through the knee and ankle joint of the left rear leg. The length of the tibia was measured (proximal to distal heads of the bone) with calipers.

A 24-hour urine collection was performed 1 day before surgery and 14 and 28 days after surgery. Urine volume, creatinine, albumin, blood urea nitrogen, total protein, and electrolyte excretion (Na^+/K^+) were determined. Each urine collection was preceded by 24 hours of metabolic cage acclimation. Twenty-four-hour endogenous creatinine clearance was used as a measure of glomerular filtration rate. On completion of final 24-hour urine collection, glomerular filtration rate was further measured by FITC-inulin. The carotid artery was implanted with a catheter for bleed. FITC-inulin (Sigma-Aldrich, Darmstadt, Germany) was injected IV at 13 mg/kg. Approximately 0.2 mL of blood was collected into heparinized tubes via the catheter at 2, 5, 10, 15, 20, 40, 60, 100, and 180 min postinjection of FITC-inulin, yielding at least 80 µL of plasma for the determination of FITC concentration by fluorescence. Plasma chemistries were performed using a Beckman Coulter (Brea, CA) AU680 chemistry analyzer.

FGF23 measurement

Plasma intact-FGF23 and plasma total-FGF23 were measured using commercially available kits (Immutopics, San Clemente, CA #60-6800 for plasma intact-FGF23, #60-6300 for plasma total-FGF23) according to the manufacturer's instructions.

Statistical analysis

Data obtained with regard to FGF23 and hypertrophy-related parameters are presented as individual points, as well as mean ± SEM, using Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). Data were analyzed using unpaired, nonparametric, 2-tailed *t* test, with or without Welch's correction depending on the obtained SD. Differences were considered significant at $P < 0.05$.

Results

5/6 Nx rats have increased FGF23 but not cardiac hypertrophy

We first characterized the rat 5/6 Nx CKD model to determine if we could recapitulate (1) increased FGF23 plasma levels and the (2) cardiac hypertrophy phenotype that would warrant further investigative studies of FGF23/FGFR4 signaling. In this study, we assessed the 2-week Nx time point as described in previously published reports and included a separate arm at 4 weeks to investigate additional phenotypic changes. At 2 and 4 weeks, animals that underwent 5/6 Nx surgery exhibited significant changes in renal function measured by plasma and urine chemistries (Fig. 1A, 1B, and 1C; Tables 1 and 2). To account for all species of FGF23, levels of intact (noncleaved) and total (cleaved and noncleaved) FGF23 were measured. Both intact and total FGF23 were significantly increased at 2 weeks but only total FGF23 levels were increased at 4 weeks (Fig. 1D and 1E). The 5/6 Nx animals had significantly decreased changes in body weight compared with shams at both 2 and 4 weeks (Fig.

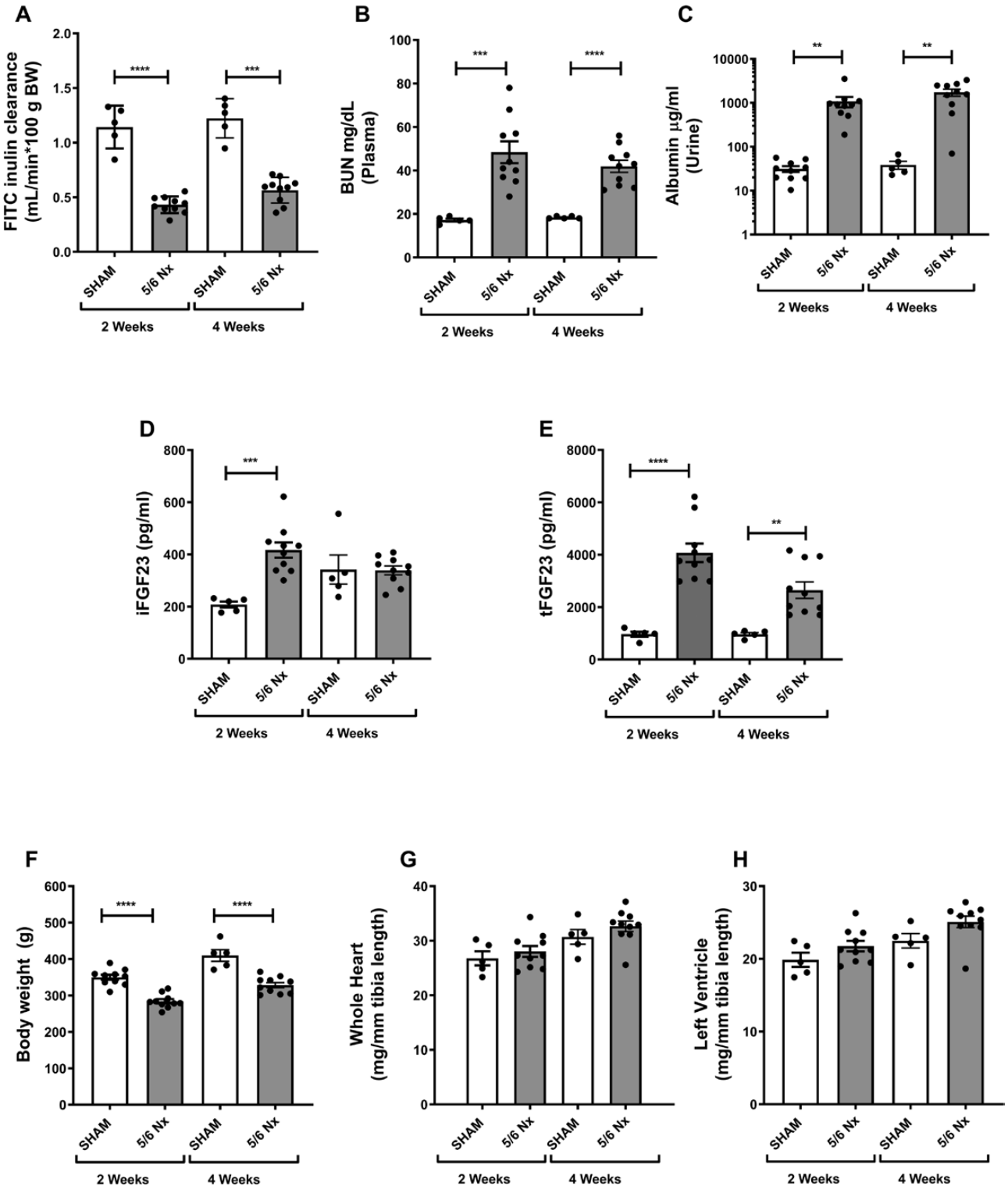

Figure 1. Cardiac hypertrophy is not induced in a rat 5/6 Nx model of CKD. Plasma and urine markers of renal function are increased after 2 and 4 weeks after 5/6 Nx: (A) FITC inulin clearance, (B) blood urea nitrogen, and (C) albumin compared with sham (n = 5). Changes to circulating levels of (D) intact and (E) total FGF23. (F) Body weight is decreased in both 2- and 4-week nephrectomized animals compared with sham. Normalized (G) cardiac whole and (H) left ventricular weights are not significantly increased after Nx compared with sham. Data presented as mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. CKD, chronic kidney disease; FGF23, fibroblast growth factor 23; Nx, nephrectomy.

Table 1. 5/6 Nx plasma clinical chemistry panel: changes to plasma chemistries at 2 and 4 weeks after Nx compared with sham

		2 week			4 week		
Plasma clinical chemistry	Units	Sham (n = 5)	5/6 Nx (n = 10)	*P* value	Sham (n = 5)	5/6 Nx (n = 10)	*P* value
Aspartate aminotransferase	(U/L)	90 ± 15	86 ± 6	NS	89 ± 5	83 ± 4	NS
Alanine phosphatase	(U/L)	34 ± 4	37 ± 2	NS	38 ± 2	39 ± 3	NS
Alkaline phosphatase	(U/L)	136 ± 17	143 ± 12	NS	124 ± 12	104 ± 6	NS
Creatinine	(mg/dl)	0.2 ± 0.0	0.7 ± 0.1	***	0.3 ± 0.0	0.7 ± 0.1	****
Total protein	(g/dL)	5.5 ± 0.1	5.4 ± 0.1	NS	6.1 ± 0.2	5.3 ± 0.1	NS
Albumin	(g/dL)	2.5 ± 0.1	2.5 ± 0.1	NS	2.8 ± 0.1	2.5 ± 0.0	NS
Globulin	(g/dL)	3.0 ± 0.1	2.8 ± 0.1	NS	3.3 ± 0.2	2.8 ± 0.0	NS
Albumin/globulin ratio	(ratio)	0.8 ± 0.0	0.9 ± 0.0	NS	0.9 ± 0.0	0.9 ± 0.0	NS
Glucose	(mg/dL)	260 ± 11	241 ± 13	NS	254 ± 5	258 ± 4	NS
Cholesterol	(mg/dL)	72 ± 3	91 ± 4	*	80 ± 9	99 ± 7	NS
Sodium	(mmol/L)	144 ± 0.4	143 ± 1	NS	145 ± 1	146 ± 1	NS
Potassium	(mmol/L)	5.8 ± 0.3	5.3 ± 0.1	NS	6.4 ± 0.4	5.3 ± 0.2	NS
Chloride	(mmol/L)	100 ± 1	97 ± 0.4	**	99 ± 1	100 ± 1	NS
Calcium	(mg/dL)	12 ± 0.1	12 ± 0.2	NS	12 ± 0.2	12 ± 0.1	NS
Phosphate	(mg/dL)	9.0 ± 0.2	9.4 ± 0.4	NS	10 ± 0.4	9.0 ± 0.3	NS
Total bilirubin	(mg/dL)	0.1 ± 0.0	0.1 ± 0.0	NS	0.1 ± 0.0	0.1 ± 0.0	NS
Glutamate dehydrogenase	(U/L)	8 ± 1	10 ± 1	NS	11 ± 1	10 ± 1	NS
Lactate dehydrogenase	(U/L)	92 ± 16	104 ± 9	NS	115 ± 22	124 ± 22	NS
Total bile acids	(µmol/L)	15 ± 5	46 ± 7	**	11 ± 4	42 ± 7	*
Blood urea nitrogen	(mg/dL)	17 ± 1	48 ± 5	***	18 ± 0.2	42 ± 3	****
Triglycerides	(mg/dL)	90 ± 12	54 ± 5	**	106 ± 21	68 ± 6	*
High-density lipoprotein	(mg/dL)	26 ± 1	36 ± 1	***	32 ± 3	40 ± 2	NS
Low-density lipoprotein	(mg/dL)	13 ± 1	12 ± 1	NS	11 ± 1	12 ± 1	NS
Nonesterified fatty acids	(mmol/L)	0.2 ± 0.0	0.2 ± 0.0	NS	0.3 ± 0.0	0.3 ± 0.0	NS
Creatine kinase	(U/L)	91 ± 10	92 ± 4	NS	97 ± 11	94 ± 11	NS
Uric acid	(mg/dL)	2.6 ± 0.4	2.5 ± 0.4	NS	4 ± 1	2.7 ± 0.1	NS

Abbreviations: NS, not significant; Nx, nephrectomy.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Table 2. 5/6 Nx urine clinical chemistry panel: changes to urine chemistries at 2 and 4 weeks post Nx compared with sham

		2 week			4 week		
Urine clinical chemistry	Units	Sham (n = 10)	5/6 Nx (n = 10)	*P* value	Sham (n = 5)	5/6 Nx (n = 10)	*P* value
Albumin	(µg/mL)	31 ± 5	1078 ± 288	**	39 ± 6	1737 ± 322	**
Albumin excretion	(mg/day)	1 ± 0.1	66 ± 19	**	1 ± 0.2	90 ± 18	**
Protein	(mg/dL)	87 ± 6	172 ± 50	NS	96 ± 2	320 ± 68	*
Protein excretion	(mg/day)	24 ± 2	104 ± 32	*	26 ± 2	163 ± 36	*
Urine	volume (mL)	29 ± 2	57 ± 4	****	28 ± 2	49 ± 3	**
Creatine	(mg/dL)	50 ± 3	18 ± 2	****	56 ± 4	26 ± 3	****
Creatine excretion	(mg/day)	14 ± 1	10 ± 0	****	15 ± 0.4	12 ± 1	**
Albumin to creatine ratio		0.1 ± 0.0	7 ± 2	**	0.1 ± 0.0	8 ± 2	**
Total protein/creatine ratio		1.8 ± 0.1	11 ± 4	*	2 ± 0.1	14 ± 4	*

Abbreviations: NS, not significant; Nx, nephrectomy.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

1F). Although we measured significant changes to FGF23 in nephrectomized animals, there were no significant changes to either whole heart weight (2-week $P = 0.70$; 4-week $P = 0.44$) or left ventricular weights normalized to tibia length (2-week $P = 0.28$; 4-week $P = 0.10$) Nx animals (Fig. 1G and 1H).

Mice fed a 2% phosphate diet for 12 weeks have increased FGF23 but do not develop cardiac hypertrophy

Comparable to the rat 5/6 Nx studies, we characterized the mouse 12-week high-phosphate diet for changes to FGF23 and cardiac hypertrophy. Circulating levels of inorganic phosphate were increased significantly (Fig. 2A) in mice fed a 2% phosphate diet compared with normal diet-fed mice. There were no significant changes to markers of renal function measured from plasma (Table 3). After 12 weeks, the body

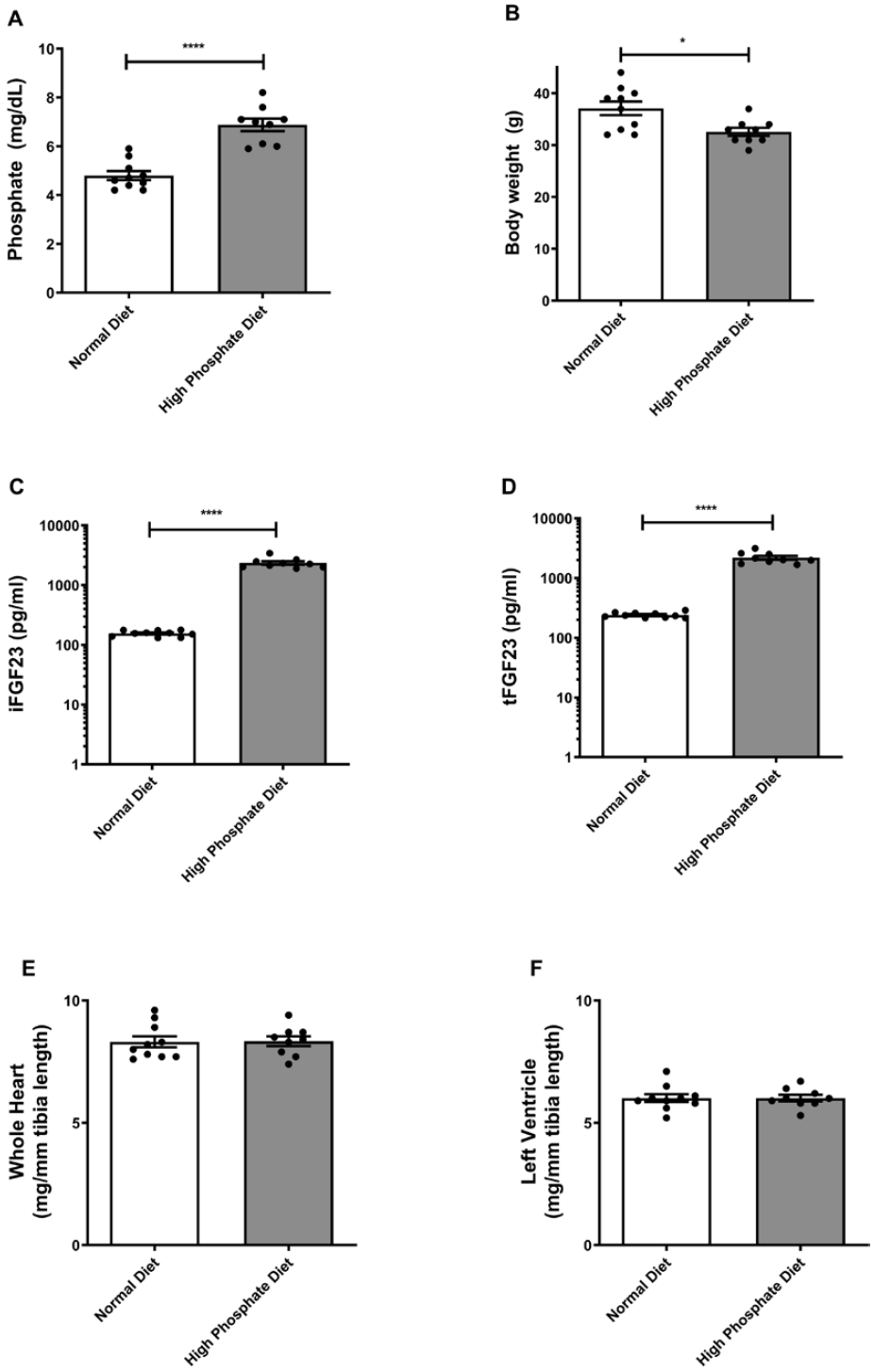

Figure 2. Cardiac hypertrophy is not induced in a mouse diet-induced model with increased FGF23. (A) Levels of circulating inorganic phosphate is increased in the high-phosphate diet fed animals compared with control diet fed animals. (B) Body weight is decreased in high-phosphate diet-fed mice. Circulating levels of (C) intact FGF23 and (D) total FGF23 are increased in the high-phosphate diet model. Normalized cardiac (E) whole and (F) left ventricular weights are not significantly increased in high-phosphate diet mice with increased FGF23. Data presented as mean ± SEM, $^{*}P < 0.05$, $^{****}P < 0.0001$, normal diet (n = 10) vs high-phosphate diet (n = 10). FGF23, fibroblast growth factor 23.

Table 3. High-phosphate-diet plasma clinical chemistry panel: changes to mouse plasma chemistries after 12 weeks of a 2% phosphate diet compared with animals fed a normal diet

Plasma clinical chemistry	Units	Normal diet	High-phosphate diet	*P* value
Aspartate aminotransferase	(U/L)	33 ± 1	36 ± 2	NS
Alanine phosphatase	(U/L)	24 ± 1	17 ± 2	**
Alkaline phosphatase	(U/L)	48 ± 1	97 ± 5	****
Creatinine	(mg/dl)	0.1 ± 0.0	0.1 ± 0.0	NS
Total protein	(g/dL)	5.1 ± 0.0	5.0 ± 0.0	NS
Albumin	(g/dL)	2.7 ± 0.0	2.7 ± 0.0	NS
Globulin	(g/dL)	2.3 ± 0.0	2.3 ± 0.0	NS
Albumin/globulin ratio	(ratio)	1.2 ± 0.0	1.2 ± 0.0	NS
Glucose	(mg/dL)	270.7 ± 5.6	248 ± 8	*
Cholesterol	(mg/dL)	114.2 ± 2.9	110 ± 2	NS
Sodium	(mmol/L)	147.3 ± 0.1	149 ± 0.3	***
Potassium	(mmol/L)	4.4 ± 0.1	4.4 ± 0.1	NS
Chloride	(mmol/L)	113 ± 0.3	112.4 ± 0.4	NS
Calcium	(mg/dL)	9.0 ± 0.0	8.9 ± 0.0	*
Phosphate	(mg/dL)	4.8 ± 0.2	6.9 ± 0.2	****
Total bilirubin	(mg/dL)	0.1 ± 0.0	0.1 ± 0.0	NS
Glutamate dehydrogenase	(U/L)	5.4 ± 0.4	4.6 ± 0.4	NS
Blood urea nitrogen	(mg/dl)	28 ± 1	27.6 ± 0.6	NS

Abbreviations: NS, not significant; Nx, nephrectomy.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

weights of high-phosphate diet-fed mice were significantly decreased compared with those fed a normal diet (Fig. 2B). At the end of 12 weeks, both intact and total circulating FGF23 levels were increased significantly by more than 10-fold in the mice fed high 2% phosphate diet compared with normal diet (Fig. 2C and 2D). Despite increased levels of circulating FGF23, there were no changes to either whole heart or left ventricular weights in the high-phosphate diet group compared with controls (Fig. 2E and 2F).

Discussion

In this report, we demonstrated that 2 different rodent models with increased circulating levels of FGF23 and contrasting levels of renal function exhibited no cardiac hypertrophy. Published findings supporting FGF23 signaling underpinning cardiac hypertrophy in the heart have been intriguing in offering a potential new link and therapeutic target to patients with CRS with high unmet medical need. Moreover, studies revealing a novel mechanism of FGF23 activating calcineurin/NFAT signaling through FGFR4 [11, 12, 31] in the absence of Klotho suggested targeting FGFR4 as a potentially attractive approach to treat patients with CRS.

We examined 2 in vivo models that exhibited increased circulating FGF23 levels to test the hypothesis that FGF23 was a driver of cardiac remodeling. Both the 5/6 Nx, which has been reported to develop significant renal dysfunction, and the 12-week high-phosphate diet, which did not cause renal impairment, showed increased intact and total FGF23 levels consistent with previous studies [11, 12, 23, 32]. However, unlike published studies, the studies here using a blinded protocol detected no increase in cardiac hypertrophy in either model. The 5/6 Nx model was extended an additional 2 weeks (double that of published studies) to ensure the lack of significant cardiac remodeling was not a function of model variability.

Although there is general consensus [21, 33-36] that increased FGF23 leads to increased cardiac morbidity and mortality, there are reports suggesting that other factors are also involved. Pastor et al showed that increased FGF23 in the absence of kidney disease does not lead to increased cardiovascular disease risk [34]. Additionally, in a mouse model of X-linked hyperphosphatemia, high circulating levels of FGF23 did not cause cardiac hypertrophy. The absence of cardiac hypertrophy observed in our 5/6 Nx and high-phosphate diet studies could be due to subtle changes in multiple factors including renin-angiotensin-aldosterone system activation and soluble Klotho levels.

FGF23 has been shown to stimulate renin-angiotensin-aldosterone system genes leading to cardiac hypertrophy in a rat 5/6 Nx model [37]. Importantly, it remains unknown if other signaling factors either directly or indirectly linked to FGF23 are responsible for cardiac hypertrophy in the 5/6 Nx and high-phosphate diet. Finally, with respect to the lack of an effect observed in these studies, previously

published 5/6 Nx studies used a different rat strain (Sprague Dawley vs Wistar), although it should be noted that the FGF23 levels and other clinical chemistry markers between strains were similar.

In conclusion, the observations noted in these studies did not reproduce the reported effects of increased FGF23 on cardiac hypertrophy.

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Author Contributions: I.M., K.A., G.G., C.S., and B.W. designed the research. I.M., K.A., and J.K. performed the experiments. I.M., K.A., and G.G. analyzed data and I.M., K.A., G.G., and J.T. wrote the manuscript.

Additional Information

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Disclosures: The authors have nothing to disclose.

Data Availability: Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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