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### Authors

Scialla, Julia J  
Lau, Wei Ling  
Reilly, Muredach P  
et al.

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## Fibroblast growth factor 23 is not associated with and does not induce arterial calcification

Julia J. Scialla, MD, MHS<sup>1,\*</sup>, Wei Ling Lau, MD<sup>2,\*</sup>, Muredach P. Reilly, MBBCh, MSCE<sup>3</sup>, Tamara Isakova, MD, MMSc<sup>1</sup>, Hsueh-Ying Yang, MS<sup>4</sup>, Matthew H. Crouthamel, PhD<sup>4</sup>, Nicholas W. Chavkin, MS<sup>4</sup>, Mahboob Rahman, MD, MS<sup>5</sup>, Patricia Wahl, PhD<sup>1</sup>, Ansel P. Amaral, BS<sup>1</sup>, Takayuki Hamano, MD, PhD<sup>6</sup>, Stephen R. Master, MD, PhD<sup>7</sup>, Lisa Nessel, MSS, MLSP<sup>6</sup>, Boyang Chai, MS<sup>6</sup>, Dawei Xie, PhD<sup>6</sup>, Radhakrishna R. Kallem, MD, MPH<sup>3</sup>, Jing Chen, MD<sup>8</sup>, James P. Lash, MD<sup>9</sup>, John W. Kusek, PhD<sup>10</sup>, Matthew J. Budoff, MD<sup>11</sup>, Cecilia M. Giachelli, PhD<sup>4</sup>, Myles Wolf, MD, MMSc<sup>1</sup>, and for the Chronic Renal Insufficiency Cohort Study Investigators

<sup>1</sup>Division of Nephrology and Hypertension, Department of Medicine, University of Miami Miller School of Medicine, Miami, FL

<sup>2</sup>Department of Medicine, Division of Nephrology, University of Washington, Seattle, WA

<sup>3</sup>Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

<sup>4</sup>Department of Bioengineering, University of Washington, Seattle, Washington

<sup>5</sup>Department of Medicine, Case Western Reserve University, Cleveland, OH

<sup>6</sup>Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

<sup>7</sup>Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

<sup>8</sup>Department of Medicine, Tulane University School of Medicine, New Orleans, LA

<sup>9</sup>Department of Medicine, University of Illinois at Chicago, Chicago, IL

<sup>10</sup>National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD

<sup>11</sup>Department of Medicine, Los Angeles Biomedical Research Institute, Torrance, CA

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Corresponding Author: Myles Wolf, MD, MMSc, 1120 NW 14<sup>th</sup> Street, CRB 819, Miami, FL, USA 33136, Phone: (305) 243-7760; Fax: (305) 243-8914; [mwolf2@med.miami.edu](mailto:mwolf2@med.miami.edu).

\* contributed equally

### Disclosure

Dr. Isakova has served as a consultant and received honoraria from Shire and Genzyme.

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## Abstract

Elevated fibroblast growth factor 23 (FGF23) is associated with cardiovascular disease in patients with chronic kidney disease. As a potential mediating mechanism, FGF23 induces left ventricular hypertrophy; however, its role in arterial calcification is less clear. In order to study this we quantified coronary artery and thoracic aorta calcium by computed tomography in 1501 patients from the Chronic Renal Insufficiency Cohort (CRIC) study within a median of 376 days (interquartile range 331 to 420 days) of baseline. Baseline plasma FGF23 was not associated with prevalence or severity of coronary artery calcium after multivariable adjustment. In contrast, higher serum phosphate levels were associated with prevalence and severity of coronary artery calcium, even after adjustment for FGF23. Neither FGF23 nor serum phosphate were consistently associated with thoracic aorta calcium. We could not detect mRNA expression of FGF23 or its co-receptor, klotho, in human or mouse vascular smooth muscle cells, or normal or calcified mouse aorta. Whereas elevated phosphate concentrations induced calcification *in vitro*, FGF23 had no effect on phosphate uptake or phosphate-induced calcification regardless of phosphate concentration or even in the presence of soluble klotho. Thus, in contrast to serum phosphate, FGF23 is not associated with arterial calcification and does not promote calcification experimentally. Hence, phosphate and FGF23 promote cardiovascular disease through distinct mechanisms.

## Keywords

phosphate; fibroblast growth factor 23; vascular calcification; vascular smooth muscle; chronic kidney disease

## Introduction

Fibroblast growth factor 23 (FGF23) is a circulating phosphaturic hormone that is elevated in patients with chronic kidney disease (CKD) and is strongly associated with mortality and cardiovascular disease.<sup>1-6</sup> To exert its primary physiologic functions, FGF23 binds to FGF receptor (FGFR)-klotho complexes in the kidney to stimulate urinary phosphate excretion and inhibit renal production of 1,25-dihydroxyvitamin D.<sup>7, 8</sup> In addition to these classic, klotho-dependent effects, FGF23 also induces left ventricular hypertrophy via FGFR-dependent, but klotho-independent, effects on cardiac myocytes.<sup>9</sup> This finding raises the possibility that elevated FGF23 levels in CKD may also contribute to other forms of subclinical cardiovascular injury, which could further explain its strong association with cardiovascular events and mortality.

Arterial calcification is a common pattern of vascular injury in CKD that begins early in the course of disease and is associated with greater risk of cardiovascular events and mortality.<sup>10-14</sup> Elevated serum phosphate is an independent risk factor for calcification.<sup>15</sup> High phosphate conditions enhance uptake of phosphate by cultured vascular smooth muscle cells (VSMC) through the type III Na-phosphate cotransporter, PiT-1. This induces osteogenic transformation and subsequent calcification.<sup>16-18</sup> Similarly, vascular deficiency of klotho may also promote calcification by increasing PiT-1-dependent phosphate uptake by VSMCs, a phenotype that can be rescued by stimulating vascular klotho expression.<sup>19, 20</sup>

Although FGF23 and klotho regulate phosphate balance, the specific effects of FGF23 on arterial calcification is less clear. Several small human studies demonstrated associations between higher levels of FGF23 and vascular calcification.<sup>21–25</sup> In contrast, calcification is not described in transgenic animals that overexpress FGF23.<sup>26–29</sup> Although this suggests that FGF23 does not induce calcification, FGF23 overexpression causes phosphate depletion, and may not generalize to human CKD in which FGF23 levels are elevated in association with normal or high serum phosphate.<sup>1</sup> In the current translational study, we aimed to determine if elevated FGF23 levels are associated with prevalence and severity of coronary and thoracic aorta calcification in a large, carefully characterized cohort of patients with CKD stages 2–4; and tested whether FGF23 affects calcification directly using experimental models that previously established the calcifying effects of phosphate.<sup>17</sup> In both the clinical and experimental analyses, we contrasted the effects of FGF23 with those of phosphate, and tested for independent effects of these novel, CKD-specific risk factors for cardiovascular disease.

## Results

We studied the association between FGF23, serum phosphate, and calcification of the coronary arteries and thoracic aorta as assessed by computed tomography (CT) in 1501 participants in the Chronic Renal Insufficiency Cohort (CRIC) Study. The study population had a mean age of  $57 \pm 12$  years, mean estimated glomerular filtration rate (eGFR) of  $47 \pm 17$  ml/min/1.73 m<sup>2</sup>, 46% of participants were female, 32% were black, 22% were Hispanic and 47% had diabetes. The median plasma FGF23 was 134.5 RU/mL (interquartile range 93.9 to 209.7 RU/mL). Higher plasma FGF23 levels were associated with decreased kidney function, other abnormalities of mineral metabolism, and traditional cardiovascular risk factors (Table 1). The median duration between measurement of biochemical parameters and assessment of calcification was 376 days (interquartile range 331 to 420 days).

### Prevalence and severity of calcification

Overall, 983 participants (65%) had prevalent coronary artery calcification (CAC) and 693 (46%) had thoracic aortic calcification (TAC), each defined as an Agatston score >0. Among those with prevalent CAC, 408 (42%) had a score  $\leq 100$ ; 233 (24%) had a score between 101–400; and 342 (35%) had a score >400. Among those with prevalent TAC (score >0), 325 (47%) had a score  $\leq 100$ ; 171 (25%) had a score between 101–400; and 197 (28%) had a score >400.

The unadjusted prevalence of CAC and TAC (scores > 0) was greater in higher quartiles of FGF23 and serum phosphate (Figure 1). Although higher plasma FGF23 was associated with greater CAC prevalence in models adjusted for demographics and kidney function (Table 2), the effect was abolished when further adjusted for traditional cardiovascular risk factors. FGF23 was not associated with CAC severity in any model (Table 2). In contrast, higher serum phosphate levels were independently associated with greater prevalence and greater severity of CAC in all adjusted models. Neither FGF23 nor phosphate was independently associated with TAC prevalence, however higher plasma FGF23 was associated with greater TAC severity in the subset with prevalent TAC (Table 2).

History of cardiovascular disease was independently associated with prevalence and severity of CAC and TAC ( $p < 0.01$  for each) and did not modify the associations between serum phosphate and prevalence and severity of CAC (data not shown). In contrast, FGF23 was associated with TAC severity only in participants with prior cardiovascular disease ( $n = 203$ ; 72% increase in TAC score per SD increase in  $\ln$  FGF23; 95% CI, 24% to 141;  $p$ -interaction = 0.04). Diabetes and severity of CKD did not modify the associations of FGF23 and serum phosphate with CAC and TAC, and there were no significant interactions between FGF23 and phosphate (data not shown). Since dietary phosphate intake alters FGF23 levels,<sup>30, 31</sup> we evaluated the association of 24-hour urinary phosphate with calcification, but found no independent associations with prevalence or severity of CAC or TAC (Supplemental Table 1).

### Sensitivity analyses

The primary results were qualitatively unchanged after excluding 74 participants who were using active vitamin D sterols or phosphate binders, when we additionally adjusted for 25-hydroxyvitamin D and 1,25-hydroxyvitamin D if available ( $n = 534$ ), and when we adjusted for iothalamate GFR in lieu of eGFR (data not shown). We repeated the primary analyses among those with less than 1 year between biochemical measurements and assessment of calcification, and among those whose renal function remained unchanged (difference in eGFR  $< 5$  ml/min/1.73m<sup>2</sup>) between measurements. In both of these sensitivity analyses, point estimates were similar to the primary analysis (Supplemental Tables 2 and 3).

To assess whether the results were robust to different modeling strategies, we fit multivariable-adjusted ordinal logistic regression models to evaluate the odds ratio of a one category (0; 1–100; 101–400; and >400) increase in CAC or TAC score. Consistent with the primary results, higher serum phosphate, but not FGF23, was associated with greater odds of being in a higher CAC category, and neither was associated with TAC using this approach (Table 3). Finally, we modeled prevalence of CAC using alternate threshold values. Similar to prevalence of CAC >0 (Table 2; Figure 2a), higher quartiles of serum phosphate, but not plasma FGF23, were associated with greater prevalence of CAC scores above versus below 100, 400 and 800 in fully adjusted models (Figures 2b–d). Unlike TAC >0 (Table 2), higher quartiles of FGF23 were associated with greater prevalence of TAC >100, 400 and 800 but the estimates of effect were highly unstable and inconsistent across the different cut points (Supplemental Figure).

### Mouse aorta and human vascular smooth muscle cells do not express FGF23 or klotho

Consistent with a recent report,<sup>32</sup> we detected expression of FGF receptors (FGFR)1 and FGFR3 in cultured human vascular smooth muscle cells (VSMCs) by reverse transcription PCR (RT-PCR, data not shown). In contrast, we did not detect expression of FGF23 or its co-receptor, klotho in human or mouse cultured VSMCs by RT-PCR (Figure 3a). To investigate whether expression might be induced in CKD, we analyzed aortas from mice after partial renal ablation and dietary phosphate loading to promote uremic vascular calcification.<sup>33</sup> FGF23 and klotho were not detected in any pooled sample of mouse aorta (2–3 aortas per pool) from 12 CKD mice, 6 of which had calcification as indicated by aortic

arch calcium content and aortic expression of the osteochondrogenic markers,<sup>33</sup> or from 7 healthy controls (data not shown).

### **FGF23 does not induce vascular calcification in vitro**

Next, we tested whether high circulating levels of FGF23 that are prevalent in CKD could induce VSMC calcification. We treated human VSMCs and mouse aortic rings that were cultured in control (1–1.4 mM phosphate) and high phosphate media (2.6 mM) with ascending concentrations of FGF23. FGF23 had no effect on the calcium content in either control or high phosphate media (Figure 3b). These results were reproduced using 2 forms of human FGF23 (intact and mutant R176Q in concentrations of up to 50 ng/mL) in cultured primary human aortic VSMCs, and supported by lack of any FGF23-mediated increase in human VSMC protein levels of phosphorylated ERK and FRS2 $\alpha$ , which are major signaling mediators downstream of klotho-dependent FGFR1 and FGFR3 activation (data not shown).<sup>34</sup>

Mouse aortic rings were pre-incubated with porcine pancreatic elastase for 24 hours prior to the 9-day calcification assay to simulate the matrix degradation that occurs in the vessels of CKD mice prior to uremic vascular calcification.<sup>33, 35</sup> Similar to the findings in human VSMCs, FGF23 with or without soluble klotho had no significant effect on calcification of mouse aortic rings cultured under high phosphate conditions (Figure 3c).

### **FGF23 with and without soluble klotho has no effect on VSMC phosphate uptake**

FGF23 regulates expression of type II sodium-dependent phosphate cotransporters in the kidney,<sup>36</sup> but its effect on the type III sodium-dependent phosphate cotransporters, PiT-1 and PiT-2, that mediate phosphate transport in VSMCs is unknown. We tested whether FGF23 regulates phosphate transport in VSMC by examining radiolabeled phosphate uptake in the presence of sodium chloride versus choline chloride to calculate sodium-dependent uptake. FGF23 (50 ng/mL) had no significant effect on phosphate uptake in the presence or absence of soluble klotho (50 ng/mL), in either mouse or human VSMCs (Figure 4).

## **Discussion**

Elevated FGF23 levels were not consistently associated with coronary or thoracic aorta calcification in patients with CKD stages 2–4 enrolled in the CRIC study. In contrast, higher levels of serum phosphate were strongly associated with CAC independent of FGF23. The lack of association of FGF23 with arterial calcification is supported by our experimental data that demonstrated no expression of FGF23 in human and mouse VSMCs, or in normal or calcified mouse aortas. Furthermore, exogenous FGF23 neither augmented phosphate uptake nor induced calcification of cultured human VSMCs regardless of the phosphate concentration of the media and whether or not exogenous soluble klotho was added. To our knowledge, these are the first data to demonstrate an adverse effect of serum phosphate on the cardiovascular system independent of and exceeding the effect of FGF23. Juxtaposed with our previous finding that FGF23 can induce left ventricular hypertrophy,<sup>9</sup> these data suggest that phosphate and FGF23 play distinct roles in the pathogenesis of cardiovascular disease in CKD and that future therapeutic strategies should target both factors.

Prior experimental literature supports a direct role of phosphate in promoting arterial calcification. Incubation of VSMCs in phosphate-enriched growth media induced differentiation into an osteoblastic phenotype with subsequent calcification,<sup>16–18</sup> and there was decreased calcification when phosphate uptake was blocked.<sup>16</sup> In a mouse model of CKD, arterial medial calcification can be induced by a high phosphate diet,<sup>33</sup> and transient elevations in serum phosphate are sufficient to drive this process.<sup>35</sup> The pathogenic role of FGF23 in calcification in vivo has been less clear in experimental settings. Calcification occurs in animal models with elevated FGF23 levels, but only in the setting of secondary FGF23 excess with hyperphosphatemia,<sup>37, 38</sup> and not in models of primary FGF23 excess in which serum phosphate is low.<sup>26–29</sup> FGF23-ablated mice and CKD rats in which FGF23 was neutralized demonstrate rapidly fatal vascular calcification associated with severe hyperphosphatemia.<sup>39, 40</sup> Collectively, these data, along with our current results, implicate phosphate rather than FGF23 as a critical factor driving calcification.<sup>41</sup>

Our results provide new insight that may help explain the seemingly contradictory results of prior human studies relating FGF23 and arterial calcification.<sup>21–25, 42, 43</sup> We observed that the association of higher plasma FGF23 with presence of CAC in minimally adjusted analyses did not persist after adjustment for traditional coronary risk factors that are associated with elevated FGF23,<sup>30</sup> and that FGF23 was not associated with CAC severity in any analysis. Unlike these generally congruent results, analyses of TAC prevalence versus TAC severity yielded divergent results. Although FGF23 was not associated with presence versus absence of TAC, and the ordinal logistic regression approach that included all participants found no association, FGF23 was associated with TAC severity among those with non-zero scores. This could be interpreted to suggest that FGF23 may promote the progression, but not the genesis, of calcification in large vessels such as the aorta. Alternatively, the isolated association of elevated FGF23 with TAC severity may represent a statistical “false positive” that resulted from the complexity of modeling “zero inflated” data, such as the Agatston score<sup>44</sup> and the relatively large number of tests we performed. Our negative experimental data support the latter conclusion. These observations emphasize that clinical studies of FGF23 and calcification are especially sensitive to how calcification is defined, which vascular bed is imaged, whether prevalence or severity is modeled, and how the large numbers of individuals with calcification scores of zero are handled methodologically.

By nesting within the CRIC study, we could adjust for a comprehensive set of clinical covariates in the largest analysis of FGF23 and calcification in CKD to date. Based on the confidence intervals we report, we can statistically exclude an association as small as a 6% increase in the prevalence and 16% increase in the severity of CAC per standard deviation increase in FGF23. Thus, if we missed an effect, it would have to be extremely small and perhaps of limited clinical relevance. Our study also has certain limitations. The lag of up to two years between biochemical measurements and assessment of calcification may have affected our ability to detect associations. However, in support of the validity of our results, the point estimates for FGF23 and phosphate were similar in the overall population and the subgroup with a shorter time lag between measurements. In addition, we were not able to fully adjust for vitamin D levels in our primary models. However, most prior studies did not adjust for vitamin D levels at all, and the subgroup analysis in which adjustment for vitamin

D had minimal effect on point estimates suggests it is unlikely that more complete adjustment would have exposed a significant association between FGF23 and CAC. Finally, in this study we were only able to study anatomic, and not functional, changes in the vasculature, which have been associated with FGF23.<sup>45</sup>

Our experimental data differ somewhat from previous studies. Unlike a recent study that reported transmembrane klotho protein expression in healthy human vessels,<sup>19</sup> we could not detect klotho expression in human VSMCs, perhaps due to different culture conditions. Furthermore, we did not identify an anti-calcification effect of FGF23 in the presence of klotho as noted previously,<sup>19</sup> however this may be due to our use of exogenous soluble klotho rather than induction of endogenous expression. In contrast to prior reports,<sup>20</sup> we did not detect decreased calcification in response to soluble klotho. This could be due to species-specific differences or the concentrations of soluble klotho that were studied. Despite their differences, it is important to note that none of the mechanistic studies has yet to identify a direct pro-calcification effect of FGF23 on VSMCs, suggesting that induction of vascular calcification is not likely to be a major disease pathway explaining the strong clinical associations between FGF23 and mortality.

Early studies that identified elevated FGF23 as a risk factor for mortality proposed that FGF23 could represent a superior biomarker of phosphate-related toxicity relative to serum phosphate.<sup>2</sup> The results of the current study advance an intermediate paradigm in which FGF23 and phosphate may exert distinct effects on the cardiovascular system, with FGF23 acting primarily on the heart and phosphate acting on the arterial tree. Furthermore, by demonstrating lack of a pro-calcification effect of FGF23, these well-powered “negative” data help to clarify the end-organ specificity of FGF23 toxicity and provide novel insight into the cardiovascular impact of disordered phosphate metabolism in CKD.

## Methods

### Study population

The CRIC Study is a prospective cohort study of 3939 adult men and women (ages 21–74 years) with mild to moderate CKD (eGFR 20–70 ml/min/1.73 m<sup>2</sup>) enrolled from seven clinical centers in the United States between 2003 and 2008, as described previously.<sup>46, 47</sup> A random subcohort of 2026 participants underwent CT for quantification of CAC and TAC within 4 years after enrollment. Twenty-nine participants were excluded from this analysis because baseline plasma samples were not available for FGF23 measurement. An additional 496 were excluded because CT was performed more than 2 years after FGF23 was assessed at the baseline visit or after dialysis was initiated, leaving a total of 1501 participants for this analysis. The study was approved by the Institutional Review Boards of participating clinical centers, and each participant provided written informed consent.

### Data collection

We measured FGF23 in stored plasma samples from the baseline visit using a second generation C-terminal assay (Immutopics, San Clemente, CA). Measurements were performed in duplicate in a central laboratory after a single thaw (coefficient of variation

<10%). We used standard assays to measure serum creatinine, albumin, calcium, phosphate, urinary albumin, and 24-hour urinary phosphate centrally. We corrected serum calcium for hypoalbuminemia.<sup>48</sup> We measured plasma parathyroid hormone using a total intact assay (Scantibodies, Santee, CA). We analyzed renal function using the CRIC estimating equation for eGFR that was derived from direct measurements of iothalamate clearance (iGFR) in a subgroup of participants.<sup>49, 50</sup>

All participants underwent two concurrent CT scans with either Electron Beam CT scanner (GE Medical Systems), or multidetector CT scanner. We used commercially available software (Neo Imagery Technologies, Inc) to quantify calcification according to the Agatston criteria.<sup>51</sup> Total CAC score was determined as the sum of scores from the left main, left anterior descending, left circumflex, and right coronary arteries. Total TAC score was determined as the sum of the scores from the ascending and descending thoracic aorta that were visible on the coronary CT scans, as has been done previously.<sup>52</sup> Final scores were calculated as the mean of the two scans.<sup>53</sup>

### Analysis of human data

We compared characteristics of the study population across quartiles of FGF23 using ANOVA, Pearson's  $\chi^2$  test, or Kruskal-Wallis test, as appropriate. For continuous analyses, we transformed FGF23 as the natural logarithm (ln), and standardized ln FGF23, serum phosphate, and 24-hour urinary phosphate to their respective standard deviations to facilitate comparison.

We estimated the association of FGF23 and serum phosphate with prevalence of CAC and TAC (score >0) using Poisson regression with robust variance estimation.<sup>54</sup> We sequentially adjusted for demographics (age, sex, race, ethnicity), kidney function (eGFR, ln-transformed urine albumin-to-creatinine ratio), clinical center, traditional cardiovascular risk factors (prior cardiovascular disease, diabetes, hypertension, hypercholesterolemia, former or current smoking, categories of body mass index), and corrected serum calcium and ln-transformed parathyroid hormone levels. Models of serum phosphate were analyzed before and after adjustment for FGF23, and models of FGF23 before and after adjustment for serum phosphate. We confirmed that the assumptions of a Poisson distribution were met by comparing our results to those obtained using negative binomial regression.

Among participants with calcification scores >0, we analyzed the association of FGF23 and serum phosphate with severity of calcification using linear regression to model the ln-transformed score with sequential adjustments as described above. Regression coefficients ( $\beta$ ) were expressed as the percent difference in CAC or TAC per 1 standard deviation increase in either ln FGF23 or serum phosphate by performing the inverse logarithm ( $e^\beta$ ).<sup>55, 56</sup> We confirmed adequate model fit using residual analysis.

To evaluate for effect modification of prevalence and severity of calcification, we performed analyses stratified by history of prior cardiovascular disease, diabetes, and CKD severity (eGFR above versus below 45 ml/min/1.73m<sup>2</sup>). We also tested for interaction between FGF23 and phosphate.

In secondary analyses, we evaluated the association of calcification with 24-hour urinary phosphate as a surrogate of dietary phosphate intake. We repeated the main analyses after excluding 74 study participants who were treated with active vitamin D sterols or phosphate binders. We adjusted for directly measured iGFR in place of eGFR in the subset of participants in whom iGFR was available (n=1034), and for 25-hydroxyvitamin D and 1,25-hydroxyvitamin D when available (n=534). We determined the association of FGF23 and serum phosphate with the prevalence of CAC and TAC using alternate threshold values (0, 100, 400 and 800), and used ordinal logistic regression to model risk of being in higher severity categories (0; 1–100; 101–400; and >400). Finally, to confirm that our results were not biased by the time elapsed between measurement of FGF23, phosphate and CT scans, we fit primary models in the population with <1 year between measurements (n=597) and among those whose eGFR was unchanged (difference  $\leq 5$  ml/min/1.73m<sup>2</sup>; n=842) between measurements. All analyses were performed using Stata 11.1 (StataCorp; College Station, TX).

### Reverse transcription PCR (RT-PCR)

Mouse aortas isolated from an experiment involving phosphate-induced uremic vascular calcification<sup>33</sup> were examined for FGF23 and klotho expression. RNA from the aortas of CKD and healthy control mice was extracted using TRIzol/chloroform followed by the RNeasy Mini Kit (Qiagen); each sample was pooled from 2 to 3 aortas. First-strand cDNA was made from total RNA using the Omniscript Reverse Transcriptase kit (Qiagen). Further, total RNA from cultured human and mouse VSMCs was extracted using the RNeasy Mini Kit, and used to make cDNA using the Omniscript Reverse Transcriptase kit (Qiagen).

We tested for human FGF23 and klotho expression using intron-spanning primers that have previously been described.<sup>32</sup> We designed intron-spanning mouse FGF23 and klotho primers, using mouse calvarial cDNA (for FGF23) and kidney cDNA (for klotho) as positive controls. Mouse FGF23 primer sequences: forward 5'-TGGGCACTGCTAGAGCCTAT-3' and reverse 5'-CTTCGAGTCATGGCTCCTGT-3'. Mouse klotho primer sequences: forward 5'-ATTGATGGCGACTACCCAGA-3' and reverse 5'-AAGGAGGAAAGCCATTGTCC-3'.

### FGF23 and klotho reagents

Intact human FGF23 and the mutant form, R176Q, of human FGF23 (P-0333) were provided by Genzyme (Cambridge, MA). Recombinant mouse FGF23 (2629-FG/CF) and klotho (1819-KL) were purchased from R&D Systems (Minneapolis, MN). In our experiments, we utilized doses of FGF23 and klotho that were in the upper end of the physiologic range (2 ng/mL) in prior human and animal studies,<sup>2, 33, 57–59</sup> and also examined higher doses (20 and 50 ng/mL) for certain experiments.

### Phosphate-induced calcification of cultured human VSMCs

Primary human aortic VSMCs were obtained from Clonetics Corporation (Palo Alto, CA). The cells were isolated, characterized, and immortalized as previously described.<sup>17</sup> Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 100 U/mL penicillin, 100 µg/mL streptomycin, 15% FBS and 1.4 mM

phosphate. Calcification was induced by supplementing the media with  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  to a final concentration of 2.6 mM phosphate. Various concentrations of native and mutant human FGF23 were tested for effect on calcification (1, 10, 20 and 50 ng/mL). Calcium content of the cultures was determined using the o-cresolphthalein complexone method and normalized to protein content as previously described.<sup>17</sup>

### Phosphate-induced calcification of cultured mouse aortic rings

Mouse aortas were harvested from 12–14 week old DBA/2J mice and perivascular fat was removed. Aortas were cut into 2–3 mm length aortic rings, which were cultured in individual wells on a 24-well plate, in DMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone, 5% FBS and 1 mM phosphate. Aortic rings were incubated in 0.01 U/mL porcine pancreatic elastase (E7885, Sigma) for 24 hours to initiate elastin breakdown, which is prevalent in the vessels of CKD mice and precedes uremic vascular calcification.<sup>35</sup> This elastase treatment protocol results in more reliable calcification of aortic rings (Lau and Giachelli, unpublished data). Calcification was induced by culture in high phosphate 2.6 mM media for 9 days, and two concentrations of mouse FGF23 with and without mouse klotho were tested (2 and 20 ng/mL). At the termination of the experiment, aortic rings were snap-frozen in liquid nitrogen, lyophilized, and decalcified with 0.6 N HCl at 37°C for 24 hours. The calcium content of the supernatant was determined colorimetrically with the o-cresolphthalein complexone kit from Teco Diagnostics (Anaheim, CA). Aortic calcium content was normalized to the dry weight of the tissue and expressed as µg Ca/mg dry weight.

### Phosphate uptake assays

Mouse primary aortic VSMCs and human aortic VSMCs were passaged in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL fungizone. The mouse and human VSMCs were seeded into 12-well and 24-well tissue culture plates respectively at  $2.5 \times 10^4$  cells per well. After 2 days of growth, the cells were washed with modified Earle's Balanced Salt Solution (EBSS) containing 146 mM sodium chloride for total uptake or 146 mM choline chloride to measure sodium independent uptake.

Cells were then incubated for 20 minutes in EBSS containing 0.05 mM inorganic phosphate and 3 µCi/ml <sup>33</sup>P orthophosphoric acid with or without FGF23 and klotho. Mouse VSMCs were incubated with 50 ng/mL mouse FGF23 and 50 ng/mL mouse klotho, while human cells were treated with 50 ng/mL mutant human FGF23 (R176Q) and 50 ng/mL mouse klotho. Each experimental condition was performed in triplicate. Finally, cells were lysed in 0.2 N NaOH for 20 minutes and neutralized with HCl. Radioactivity was measured using a liquid scintillation counter and was normalized to protein content (Pierce Micro BCA Protein Assay Kit, Thermo Scientific, Rockford, IL).

### Analysis of experimental data

Statistical analyses were performed using SPSS software v16.0 (SPSS, Chicago, IL). Group means were compared using one-way ANOVA with Bonferroni post-hoc analysis, with significance set at  $p < 0.05$ .

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The CRIC Study Investigators includes: Lawrence J. Appel, MD, MPH, Harold I. Feldman, MD, MSCE, Alan S. Go, MD, Jiang He, MD, PhD, John W. Kusek, PhD, James P. Lash, MD, Akinlolu Ojo, MD, PhD, Mahboob Rahman, MD, and Raymond R. Townsend, MD.

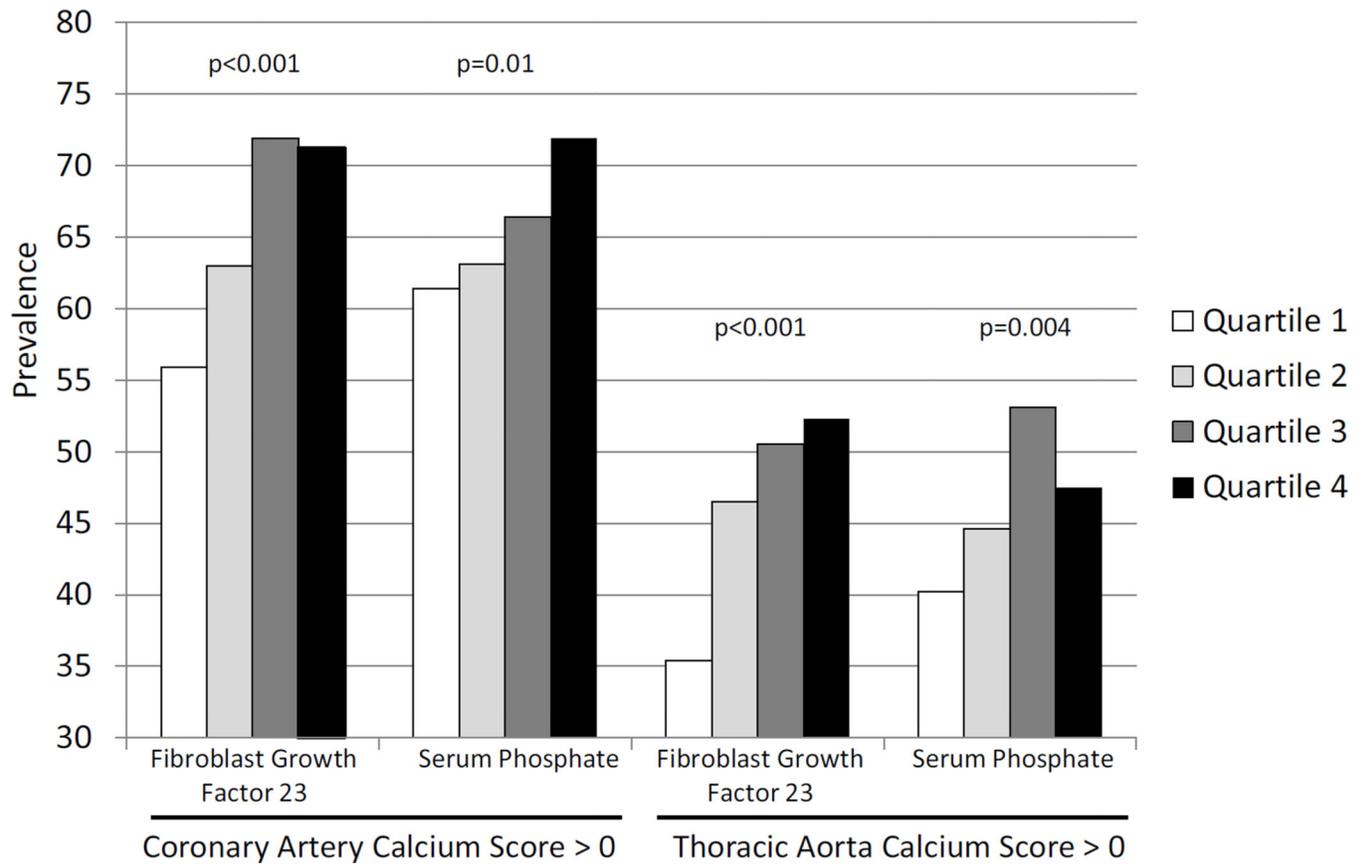
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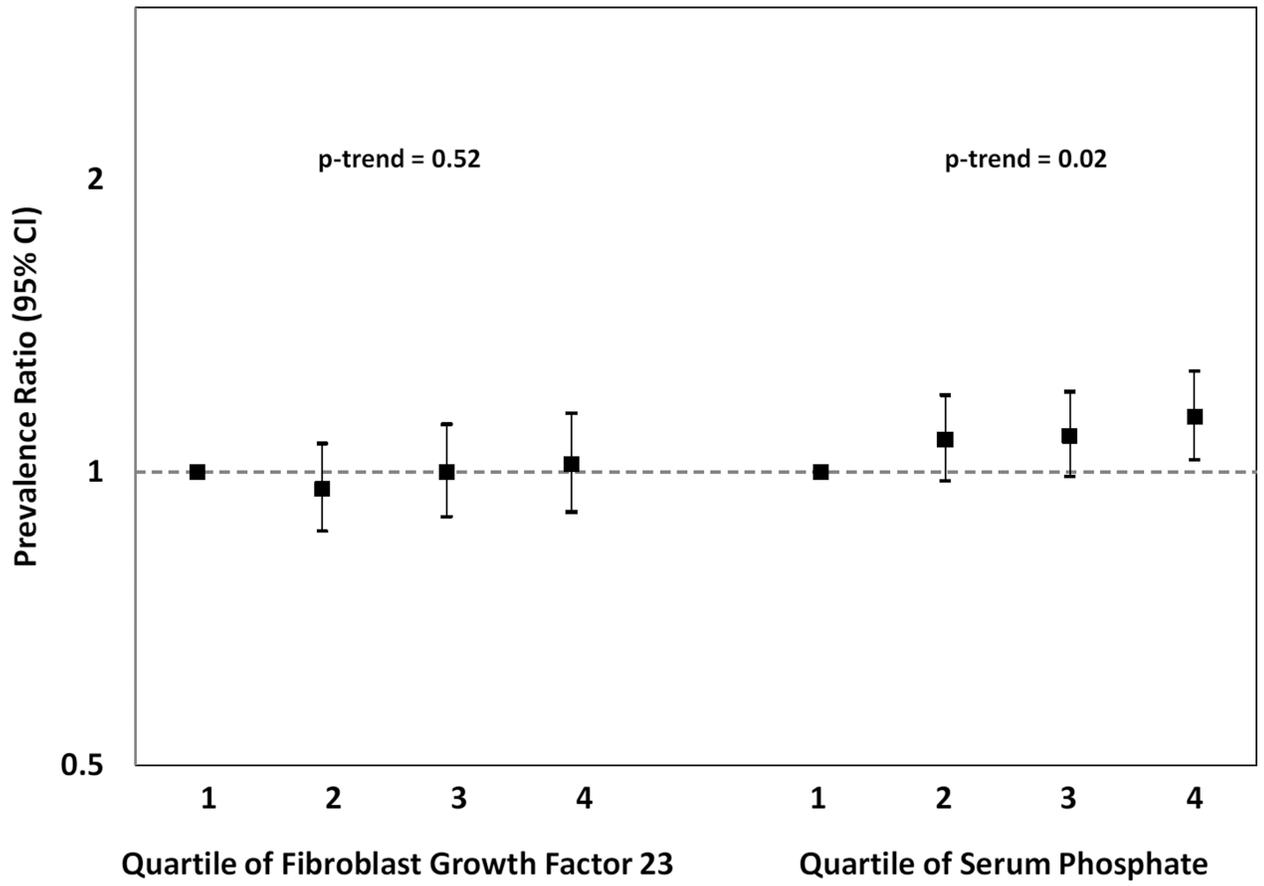
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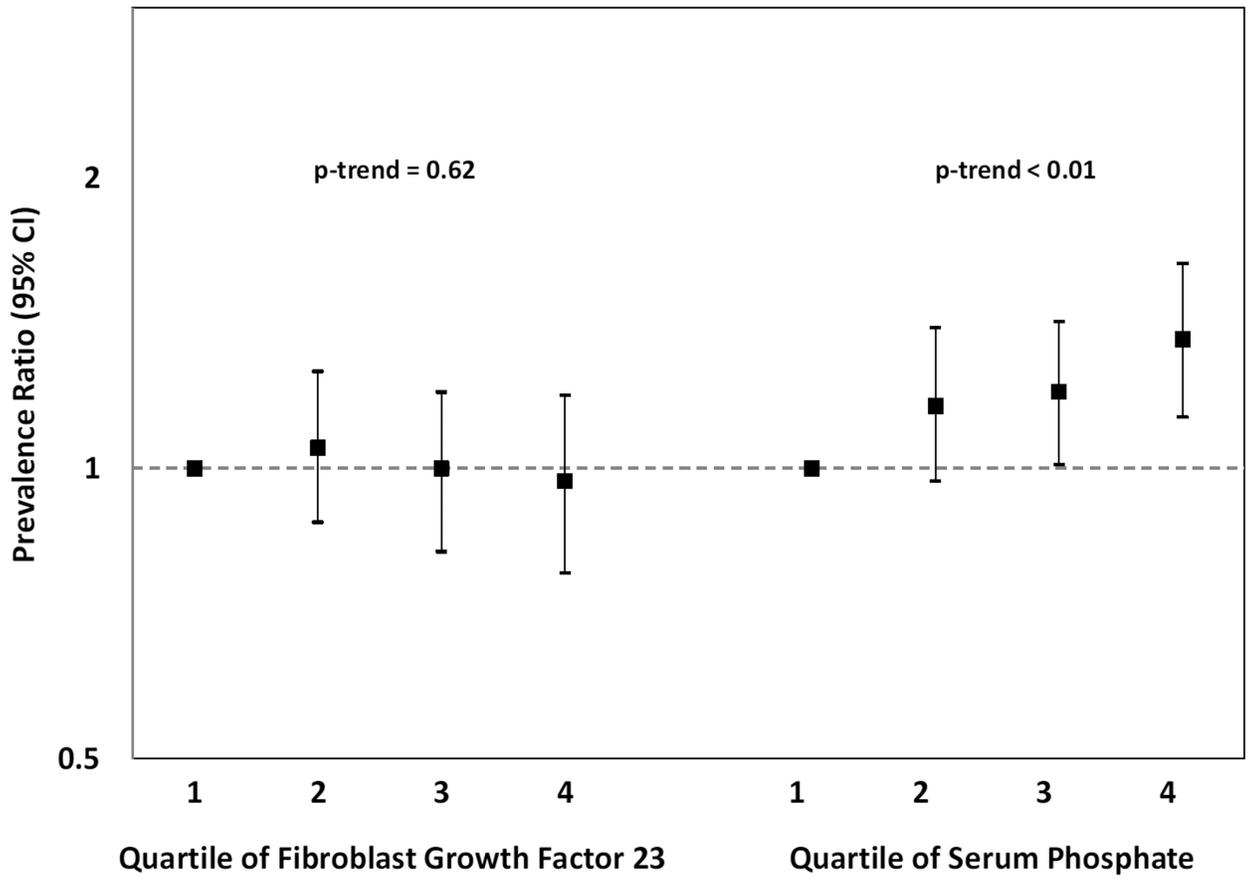


**Figure 1.** Prevalence of coronary artery calcium and thoracic aorta calcium scores > 0 across quartiles of plasma fibroblast growth factor 23 (n=1501) and serum phosphate (n=1470).

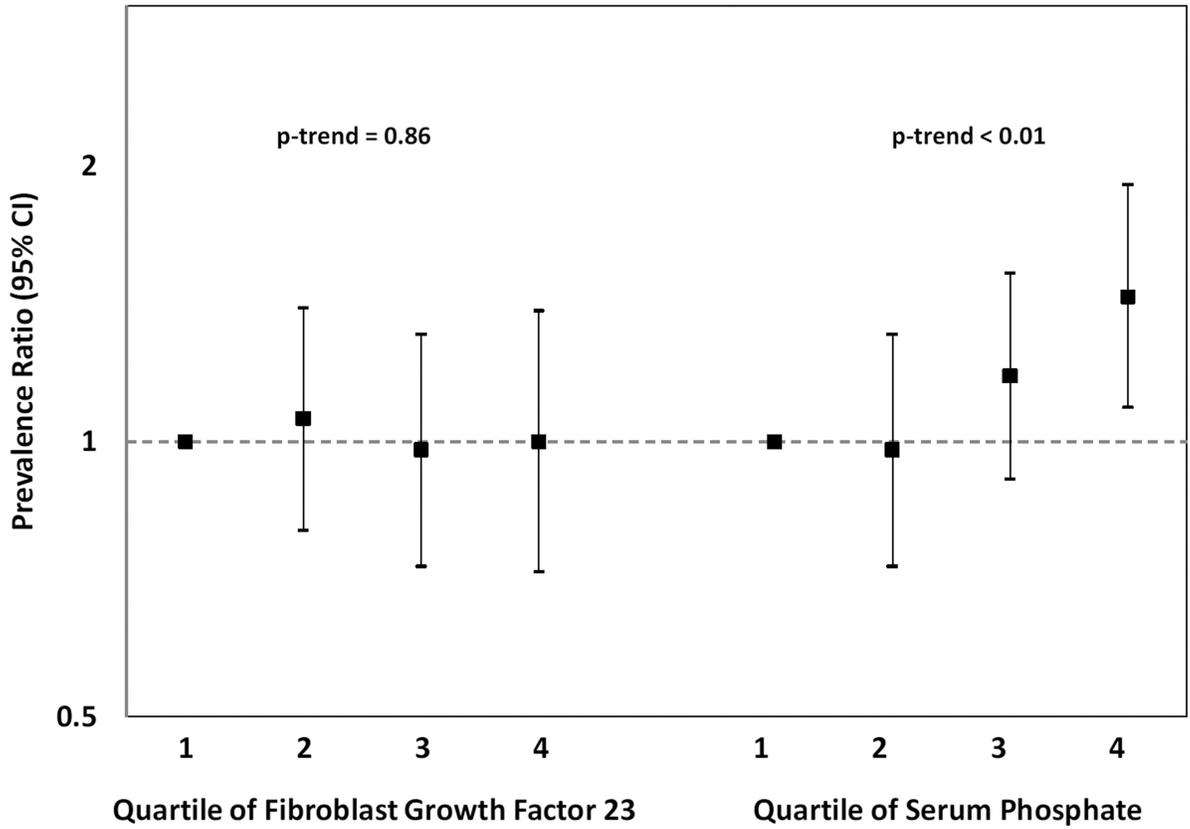
a . Adjusted prevalence ratio of CAC >0 by quartiles of fibroblast growth factor 23 and serum phosphate



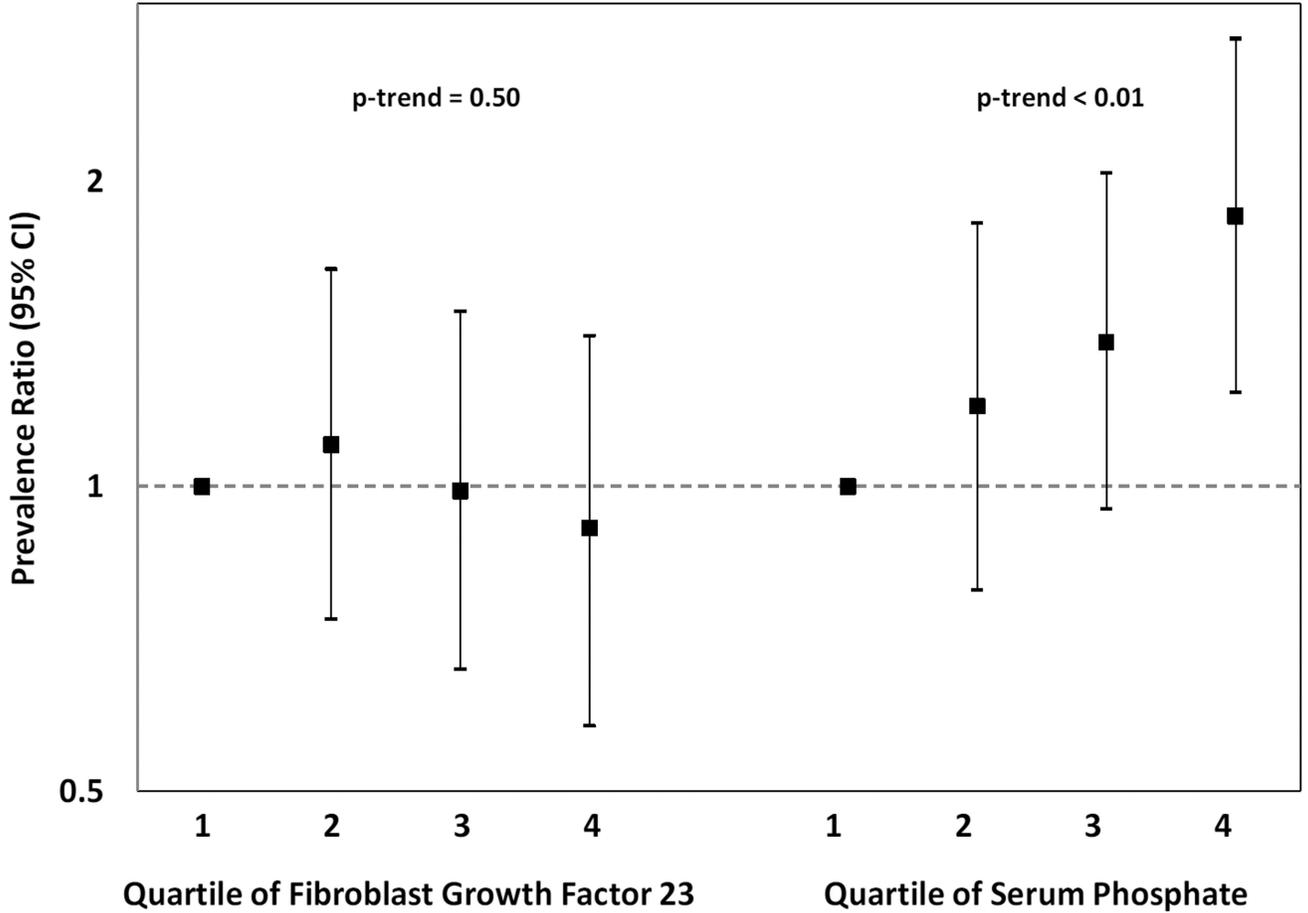
**b. Adjusted prevalence ratio of CAC > 100 by quartiles of fibroblast growth factor 23 and serum phosphate**



**c. Adjusted prevalence ratio of CAC > 400 by quartiles of fibroblast growth factor 23 and serum phosphate**

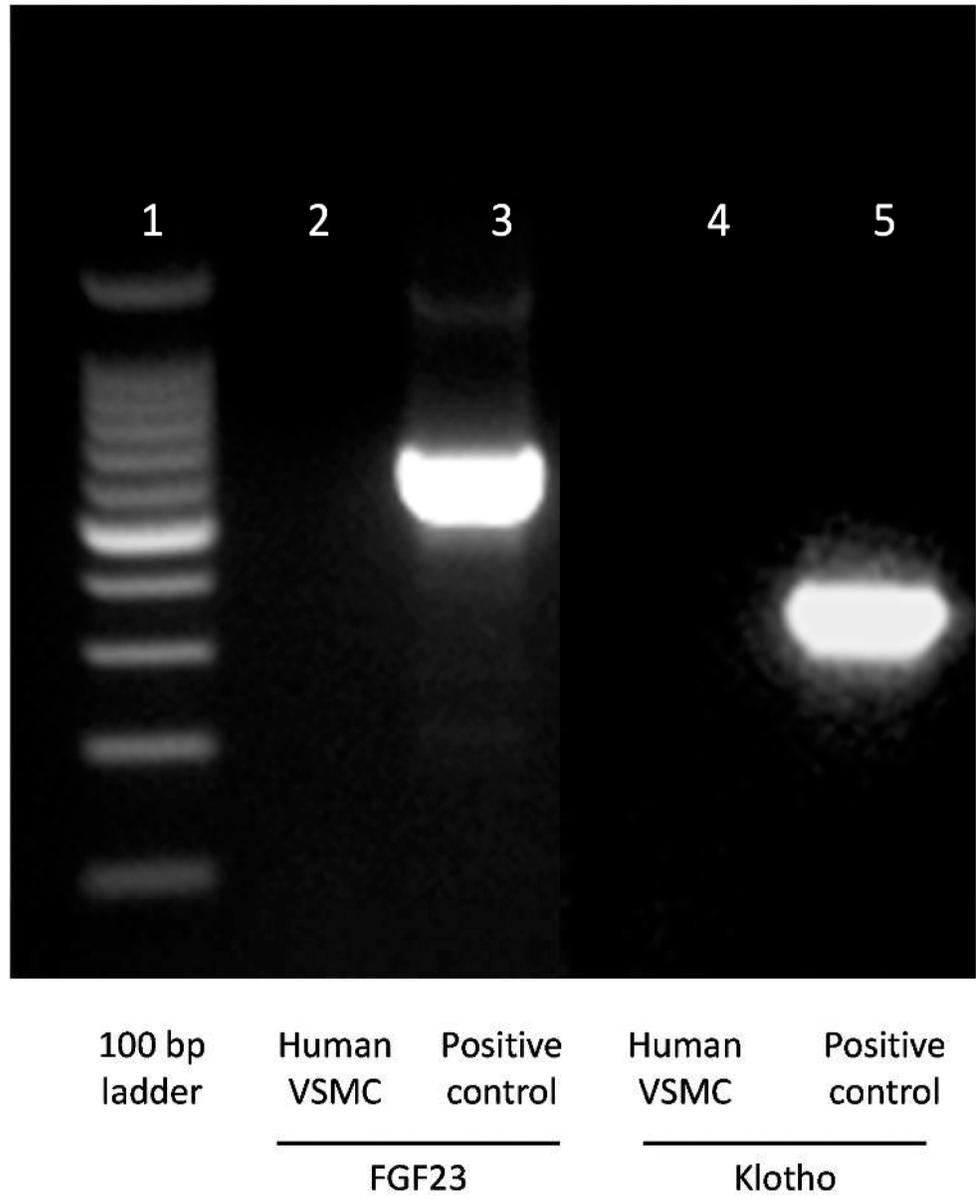


d. Adjusted prevalence ratio of CAC > 800 by quartiles of fibroblast growth factor 23 and serum phosphate

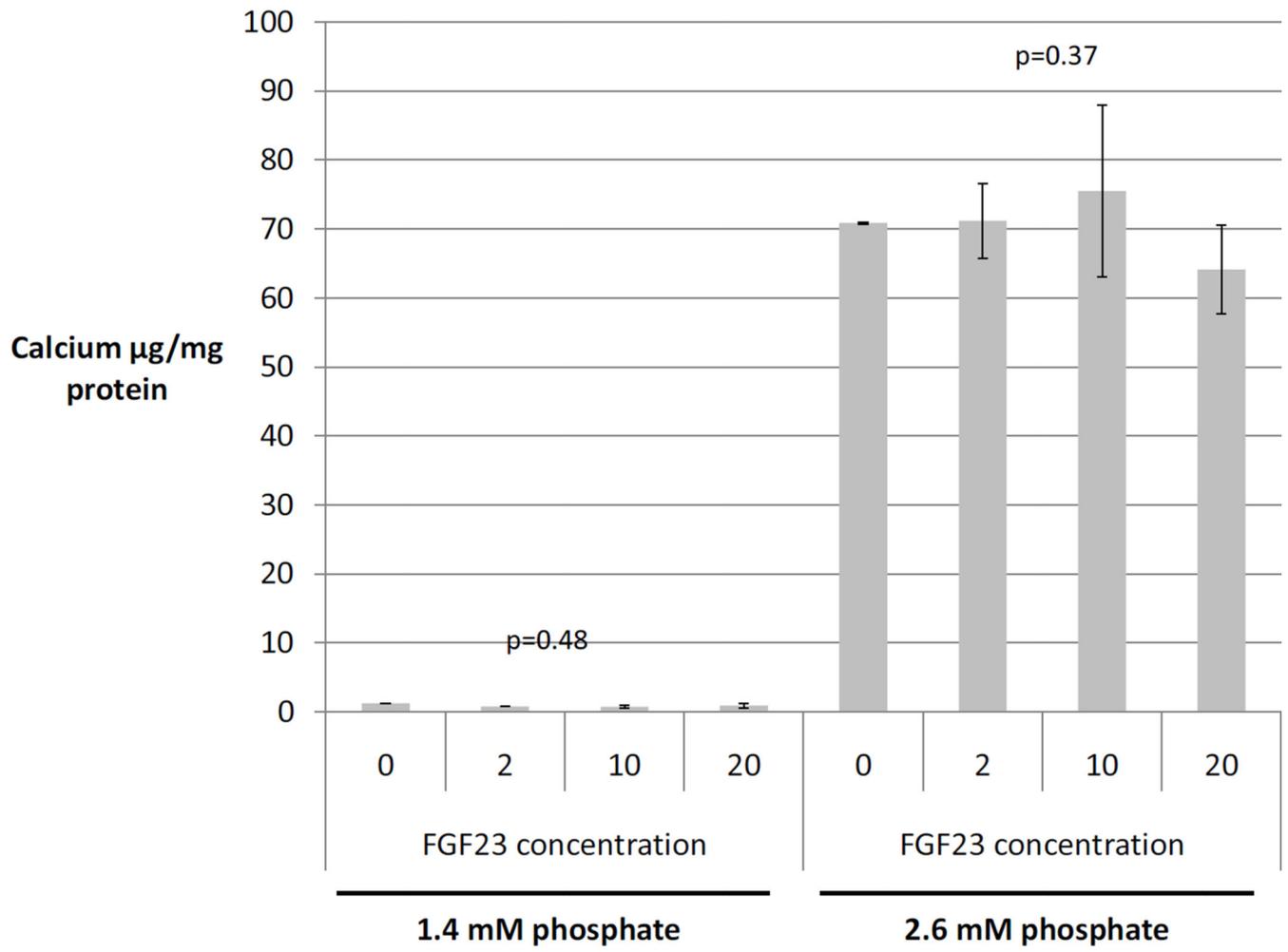


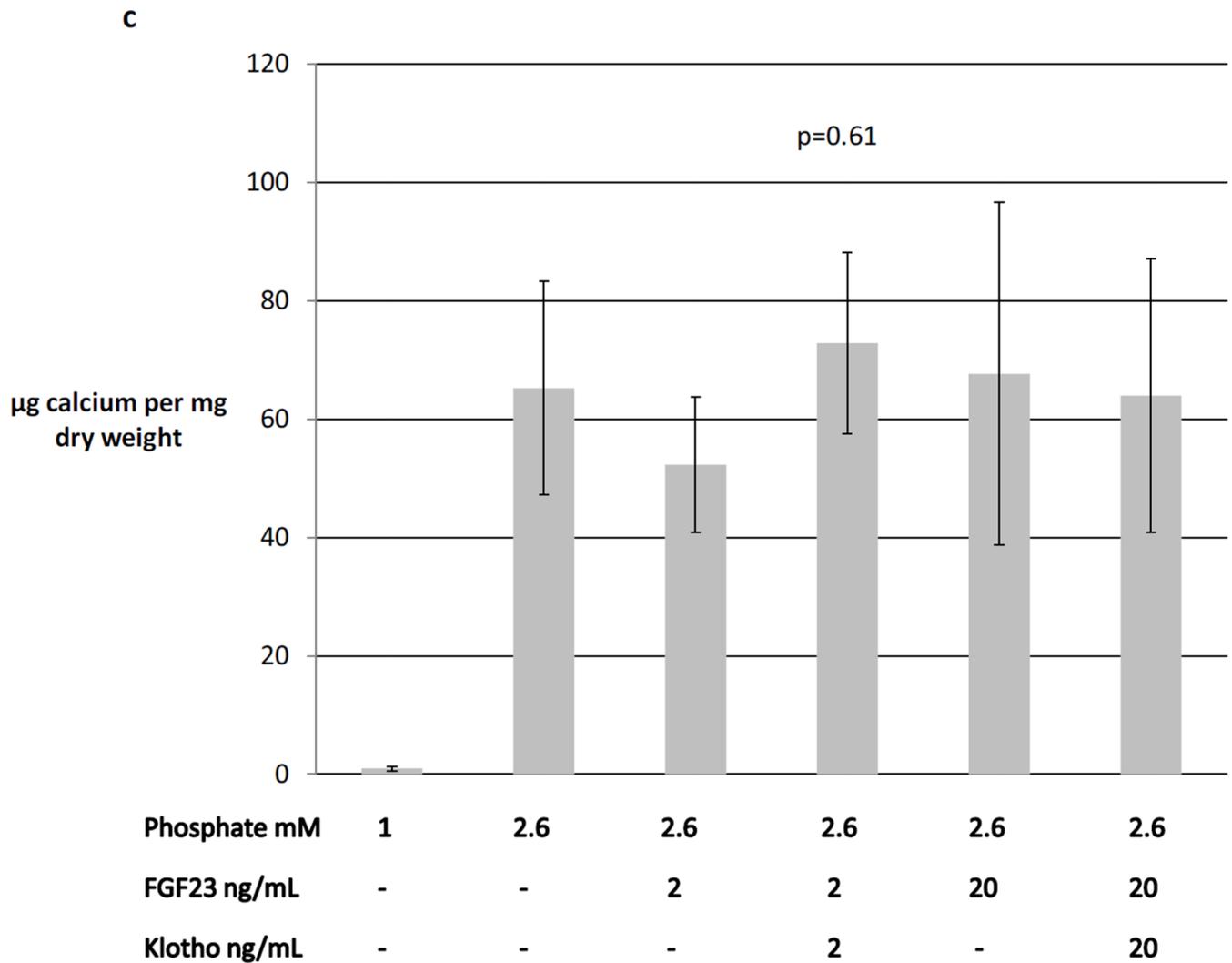
**Figure 2.** Adjusted prevalence ratios (black squares) and 95% confidence intervals (vertical bars) of coronary artery calcium (CAC) score greater than threshold values of (A) >0; (B) >100; (C) >400; and (D) >800. Results are presented by quartiles of plasma fibroblast growth factor 23 (FGF23) and serum phosphate with quartile 1 serving as the reference group. All models are adjusted for age, sex, race, ethnicity, eGFR, urine albumin-to-creatinine ratio, prior cardiovascular disease, diabetes, smoking, hypertension, hypercholesterolemia, body mass index, parathyroid hormone, corrected serum calcium, and clinical center. Models of FGF23 were additionally adjusted for serum phosphate. Models of serum phosphate were additionally adjusted for FGF23. P-values represent tests of trend across quartiles.

**a**



**b**



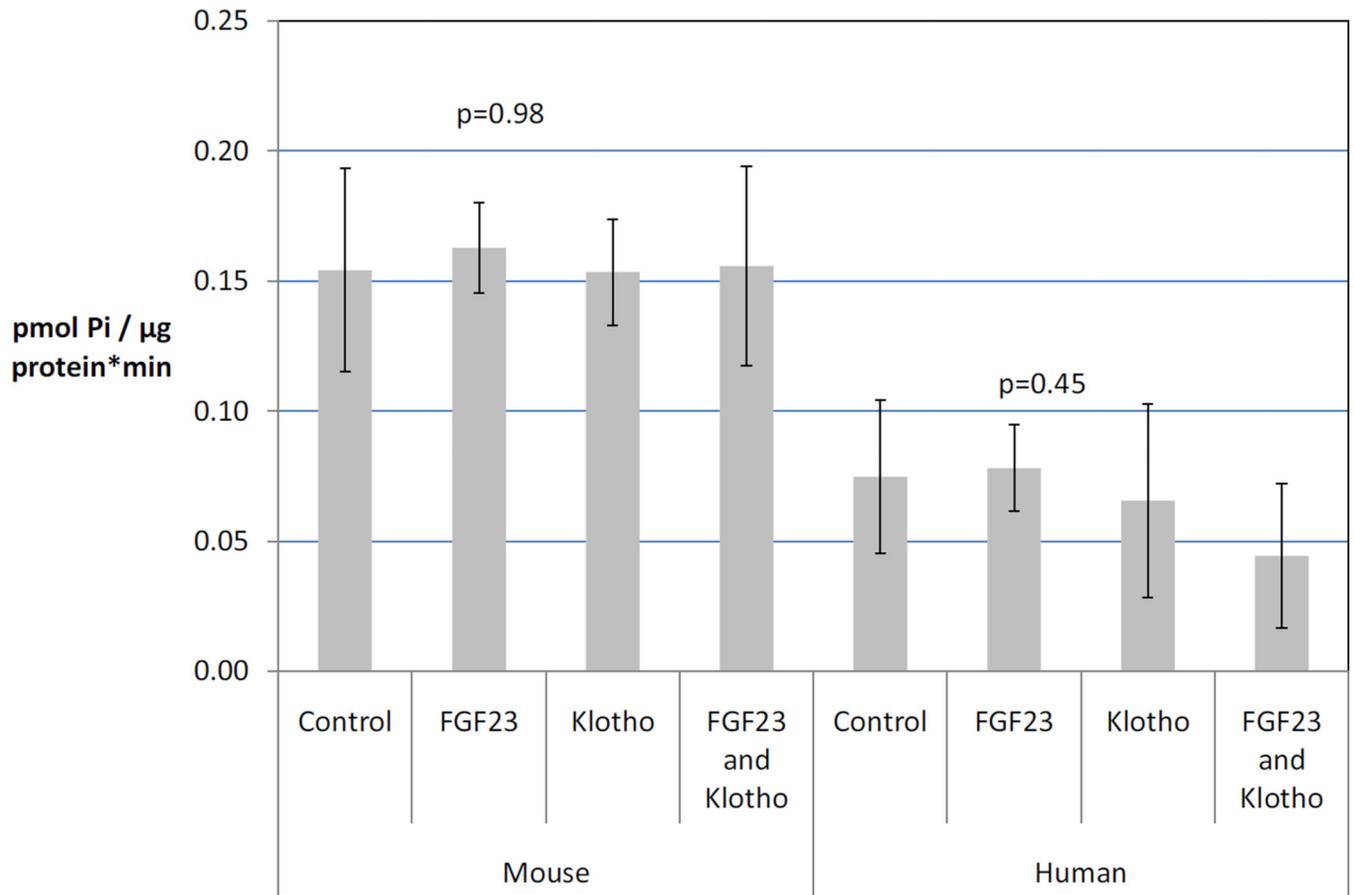


**Figure 3.**

(A) RT-PCR did not detect fibroblast growth factor 23 (FGF23) or klotho cDNA in human vascular smooth muscle cells (VSMCs), using primer sets that have previously been described.<sup>32</sup> FGF23 positive control from plasmid cDNA (expected band size 649 bp), and klotho positive control from human kidney cDNA (expected band size 349 bp).

(B) In cultured human VSMCs, FGF23 did not induce calcification under control conditions (1.4 mM phosphate) and did not augment calcification under high-phosphate conditions (2.6 mM phosphate). Data are mean  $\pm$  s.d. and p-values for the two phosphate conditions are shown.

(C) FGF23 with or without soluble klotho did not affect phosphate-induced calcification of mouse aortic rings ( $n = 5$  per group). Data are mean  $\pm$  s.d. and p-value for the high-phosphate groups is shown.



**Figure 4.** Fibroblast growth factor 23 (50 ng/mL) with or without klotho (50 ng/mL) had no significant effect on sodium-dependent phosphate uptake in both mouse and human VSMCs. Data expressed as mean  $\pm$  s.d. and p-values for the human and mouse data sets are presented.

**Table 1**  
 Characteristics of the study population by quartiles of plasma fibroblast growth factor 23

Characteristic mean ± SD or n (%)	Fibroblast Growth Factor 23 (RU/ml)				p*
	Quartile 1 1.8-93.8 N=376	Quartile 2 93.9-134.4 N=376	Quartile 3 134.5-209.6 N=374	Quartile 4 209.7-5354.8 N=375	
<u>Demographics</u>					
Age (years)	54 ± 12	57 ± 12	59 ± 11	57 ± 11	<0.01
Female sex	145 (38.6)	167 (44.4)	169 (45.2)	208 (55.5)	<0.01
Race					
White	192 (51.0)	176 (46.8)	180 (48.1)	155 (41.3)	0.01
Black	119 (31.7)	123 (32.7)	99 (26.5)	136 (36.3)	
Other	65 (17.3)	77 (20.5)	95 (25.4)	84 (22.4)	
Hispanic ethnicity	55 (14.6)	67 (17.8)	96 (25.7)	105 (28.0)	<0.01
<u>Traditional CV Risk Factors</u>					
Prior CVD	50 (13.3)	75 (20.0)	90 (24.1)	133 (35.5)	<0.01
Diabetes	112 (29.8)	157 (41.8)	208 (55.6)	230 (61.3)	<0.01
Former/current smoker	170 (45.2)	188 (50.0)	180 (48.1)	202 (53.9)	0.12
Body mass index (kg/m <sup>2</sup> )					
<25	81 (21.6)	77 (20.5)	53 (14.2)	49 (13.1)	<0.01
25-29.9	122 (32.5)	125 (33.2)	128 (34.2)	83 (22.1)	
30-39.9	151 (40.3)	145 (38.6)	154 (41.2)	180 (48.0)	
40	21 (5.6)	29 (7.7)	39 (10.4)	63 (16.8)	
Hypertension	270 (71.8)	313 (83.2)	337 (90.1)	349 (93.1)	<0.01
Hypercholesterolemia	248 (66.0)	275 (73.1)	311 (83.4)	307 (81.9)	<0.01
<u>Kidney Function</u>					
Estimated GFR (ml/min/1.73m <sup>2</sup> )	60.3 ± 17.1	50.8 ± 15.6	41.6 ± 11.7	34.8 ± 13.0	<0.01
CKD stage					
2 (eGFR 60 ml/min/1.73m <sup>2</sup> )	181 (48.1)	95 (25.3)	20 (5.4)	9 (2.4)	
3a (eGFR 45-59 ml/min/1.73m <sup>2</sup> )	124 (33.0)	136 (36.3)	121 (32.4)	64 (17.1)	
3b (eGFR 30-44 ml/min/1.73m <sup>2</sup> )	65 (17.3)	114 (30.4)	170 (45.5)	153 (40.8)	
4/5 (eGFR <30 ml/min/1.73m <sup>2</sup> )	6 (1.6)	30 (8.0)	63 (16.8)	149 (39.7)	

Characteristic mean ± SD or n (%)	Fibroblast Growth Factor 23 (RU/ml)				p*
	Quartile 1 1.8–93.8 N=376	Quartile 2 93.9–134.4 N=376	Quartile 3 134.5–209.6 N=374	Quartile 4 209.7–5354.8 N=375	
Urinary ACR (mg/g) <sup>†</sup>	15 (5, 140)	31 (6, 341)	101 (11, 782)	224 (29, 1632)	<0.01
<30	215 (59.4)	173 (48.7)	145 (40.2)	89 (25.0)	<0.01
30–299	78 (21.6)	88 (24.8)	77 (21.3)	100 (28.1)	
300–999	45 (12.4)	47 (13.2)	65 (18.0)	49 (13.8)	
1000	24 (6.6)	47 (13.2)	74 (20.5)	118 (33.2)	
<b>Mineral Metabolism Parameters</b>					
Corrected serum calcium (mg/dl)	9.18 ± 0.40	9.18 ± 0.44	9.27 ± 0.48	9.32 ± 0.48	<0.01
Serum phosphate (mg/dl)	3.39 ± 0.53	3.63 ± 0.56	3.81 ± 0.62	4.08 ± 0.81	<0.01
Parathyroid hormone (pg/ml) <sup>‡</sup>	41 (29, 61)	49 (32, 74)	54 (38, 87)	77 (45, 135)	<0.01
Parathyroid hormone >65 pg/ml	78 (21.1)	116 (31.4)	147 (39.7)	219 (59.2)	<0.01
Use of active vitamin D	5 (1.3)	4 (1.1)	13 (3.5)	15 (4.0)	0.02
Use of phosphate binders	5 (1.3)	7 (1.9)	14 (3.8)	19 (5.1)	<0.01

\* p-value by anova or kruskal-wallis test (continuous variables) and Chi-squared (categorical variables)

<sup>‡</sup> presented as median (IQR)

CVD, cardiovascular disease; GFR, glomerular filtration rate; CKD, chronic kidney disease; ACR, albumin to creatinine ratio

**Table 2**  
Adjusted association of fibroblast growth factor 23 (FGF23) and serum phosphate with prevalence and severity of coronary artery and thoracic aorta calcification

Model*	Coronary Artery Calcification			Thoracic Aorta Calcification		
	Prevalence Prevalence ratio	Severity <sup>†</sup> % difference in CAC	p	Prevalence Prevalence ratio	Severity <sup>†</sup> % difference in TAC	p
<b>Demographic, kidney function adjusted</b>						
Ln FGF23 (per SD <sup>‡</sup> )						
-Phosphate (n=1434)	1.05 (1.01 to 1.09)	5% (-10% to 22%)	0.02	1.02 (0.97 to 1.08)	30% (8% to 56%)	<0.01
+Phosphate (n=1404)	1.04 (1.00 to 1.08)	1% (-13% to 18%)	0.04	1.02 (0.96 to 1.08)	34% (11% to 62%)	<0.01
Phosphate (per SD <sup>‡</sup> )						
-FGF23 (n=1404)	1.09 (1.05 to 1.13)	37% (19% to 58%)	<0.01	1.07 (1.01 to 1.13)	13% (-5% to 35%)	0.16
+FGF23 (n=1404)	1.08 (1.05 to 1.13)	37% (18% to 58%)	<0.01	1.06 (1.00 to 1.13)	9% (-8% to 30%)	0.33
<b>+ Traditional risk factors</b>						
Ln FGF23 (per SD <sup>‡</sup> )						
-Phosphate (n=1432)	1.02 (0.98 to 1.06)	0% (-14% to 16%)	0.24	1.00 (0.94 to 1.06)	24% (4% to 48%)	0.02
+Phosphate (n=1402)	1.02 (0.98 to 1.06)	-2% (-16% to 14%)	0.32	1.00 (0.94 to 1.05)	29% (7% to 55%)	<0.01
Phosphate (per SD <sup>‡</sup> )						
-FGF23 (n=1402)	1.06 (1.02 to 1.10)	26% (10% to 46%)	<0.01	1.05 (0.99 to 1.11)	12% (-6% to 34%)	0.19
+FGF23 (n=1402)	1.06 (1.02 to 1.10)	27% (10% to 46%)	<0.01	1.05 (0.99 to 1.11)	9% (-9% to 29%)	0.35
<b>+ Corrected calcium and PTH</b>						
Ln FGF23 (per SD <sup>‡</sup> )						
-Phosphate (n=1384)	1.02 (0.99 to 1.07)	2% (-12% to 18%)	0.22	0.99 (0.94 to 1.05)	31% (9% to 58%)	<0.01
+Phosphate (n=1372)	1.02 (0.98 to 1.06)	-1% (-15% to 16%)	0.36	0.99 (0.94 to 1.05)	31% (9% to 58%)	<0.01
Phosphate (per SD <sup>‡</sup> )						
-FGF23 (n=1372)	1.06 (1.02 to 1.10)	26% (9% to 46%)	<0.01	1.04 (0.98 to 1.11)	12% (-6% to 34%)	0.21
+FGF23 (n=1372)	1.06 (1.02 to 1.10)	26% (9% to 46%)	<0.01	1.04 (0.99 to 1.11)	8% (-10% to 29%)	0.40

\* Demographic and kidney function adjusted model includes age, sex, race, Hispanic ethnicity, eGFR, ln-transformed urine albumin-to-creatinine ratio and clinical center + traditional risk factor model additionally adjusted for prior cardiovascular disease, diabetes, hypertension, hypercholesterolemia, smoking, and categories of body mass index + corrected calcium and PTH model additionally adjusted for corrected serum calcium and ln-transformed PTH

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+Phosphate is additionally adjusted for serum phosphate

+FGF23 is additionally adjusted for ln-transformed FGF23

<sup>†</sup> among those with non-zero Agatston score (n=920 for CAC; 653 for TAC in demographic and kidney function adjusted model including both serum phosphate and FGF23)

<sup>‡</sup> SD of Ln FGF23=0.72; SD of phosphate=0.69

**Table 3**

Adjusted association of fibroblast growth factor 23 (FGF23) and serum phosphate with categories of coronary artery calcium (CAC) and thoracic aorta calcium (TAC) scores using ordinal logistic regression (n=1384)

Model*	Odds ratio for 1 unit increase in CAC category <sup>†</sup>	p	Odds ratio for 1 unit increase in TAC category <sup>†</sup>	p
Ln FGF23 (per SD <sup>‡</sup> )				
-Phosphate	1.05 (0.93–1.20)	0.43	1.07 (0.94–1.21)	0.33
+Phosphate	1.02 (0.90–1.16)	0.74	1.06 (0.93–1.21)	0.38
Phosphate (per SD <sup>‡</sup> )				
-FGF23	1.29 (1.14–1.46)	<0.01	1.12 (0.98–1.27)	0.10
+FGF23	1.29 (1.13–1.46)	<0.01	1.11 (0.97–1.26)	0.13

\* adjusted for age, sex, race, Hispanic ethnicity, eGFR, ln-transformed urine albumin-to-creatinine ratio, prior cardiovascular disease, diabetes, hypertension, hypercholesterolemia, smoking, body mass index, corrected serum calcium, ln-transformed PTH and clinical center

+Phosphate is additionally adjusted for serum phosphate

+FGF23 is additionally adjusted for ln-transformed FGF23

<sup>†</sup> Categories defined as: category 1, score=0; category 2, score 1–100; category 3, score 101–400; category 4, score > 400.

<sup>‡</sup> SD of Ln FGF23=0.72; SD of phosphate=0.69