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Clinical Study

Fibroblast Growth Factor 23 Predicts Left Ventricular Mass and Induces Cell Adhesion Molecule Formation

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Elevated FGF-23 is a predictor of mortality and is associated with LVH in CKD. It may be a biomarker or a direct toxin. We assessed the relationship between FGF-23 and LVH in CKD using CMRI. In vitro we studied the effect of phosphate, FGF-23, and Klotho on E-selectin and VCAM production in HUVECs. FGF-23 concentration correlates negatively with eGFR and positively with LVMI. FGF-23 was an independent predictor of LVH in CKD. E-selectin and VCAM production was elevated in HUVECs cultured in high phosphate with FGF-23 or Klotho. This effect was attenuated in cells exposed to both FGF-23 and Klotho. FGF-23 is an independent predictor of LVH as measured by CMRI. We show preliminary data which supports that FGF-23 is toxic resulting in activation of the vascular endothelium. We do not prove causality with elevated FGF-23 and LVH. Further research should ascertain if lowering levels of FGF-23 translates to improved clinical outcomes.

1. Introduction

The excess risk of cardiovascular disease and death in those with chronic kidney disease (CKD) compared with the general population is yet to be adequately explained [1–5]. It is well documented that patients with CKD have endothelial dysfunction and it is likely that excess cardiovascular risk is secondary to a combination of traditional cardiovascular risk factors, including hypertension and diabetes mellitus, and other "nontraditional" risk factors which may be unique to the CKD population [2]. The abnormalities which arise as a result of disordered bone mineral metabolism: hyperphosphataemia, hyperparathyroidism, and hypovitaminosis D are examples of "non-traditional" cardiovascular risk factors [6–9]. Each has been linked independently with increased cardiovascular and all-cause mortality. The mechanisms are poorly described but, in animal studies, there is evidence that all can induce vascular calcification and endothelial dysfunction [9, 10]. Whilst there is some evidence that oral phosphate binders may lower levels of vascular calcification and improve survival, there are no placebo controlled trials and there is no evidence to link lower phosphate levels with improved cardiovascular outcomes [11].

Fibroblast growth factor-23 (FGF-23), a phosphaturic hormone, has emerged as an essential player in serum phosphate and vitamin D regulation. It is secreted predominantly by bone and appears to require Klotho, a transmembrane protein, in order to exert its effects. FGF-23 potently lowers serum phosphate levels by inducing renal phosphate wasting; FGF-23 inhibits the sodium phosphate cotransporter types IIa and IIc (NPT2a/2c) in the proximal tubule. It also reduces renal expression of CYB27B1 by diminishing 1.25(OH)2D3 synthesis and FGF-23 reduces parathyroid hormone (PTH) secretion [12, 13]. FGF-23 is a sensitive biomarker of abnormal renal phosphate handling and levels rise earlier, and to a greater extent, than serum phosphate in progressive CKD [14]. Levels are elevated, often more than 1000-fold, in patients with end-stage renal disease (ESRD) and in advanced CKD, hyperphosphataemia develops despite increased levels of FGF-23. This reduced responsiveness to

FGF-23 may reflect the reduction in the number of intact nephrons, and the resulting reduced expression of Klotho on distal convoluted cells [15].

Elevated FGF-23 is itself predictive of adverse outcome including premature mortality, CKD progression, vascular dysfunction, and left ventricular hypertrophy [9, 16–18]. However, it is unclear whether at elevated levels FGF-23 exerts directly toxic effects on vascular and cardiac cells or whether it is simply a biomarker for these disease states. Cardiac magnetic resonance imaging (CMRI) scanning is acknowledged as the gold standard for left ventricular mass (LVM) measurement and is superior to echocardiography [19]. The relationship between FGF-23 and LVH, measured by CMRI has not been studied previously. The aim of this study was to assess the relationship between FGF-23 and LVH using CMRI in patients with CKD stages 3 and 4. We also assessed the effects of phosphate, FGF-23 and Klotho on the expression of E-selectin and VCAM in human endothelial umbilical vein cells (HUVECs), as markers of endothelial dysfunction associated with accelerated cardiovascular disease in CKD [20, 21].

2. Materials and Methods

2.1. Study Design, Setting, and Participants. The Renal Unit at the Western Infirmary, Glasgow provides renal services to 2.8 million patients in the West of Scotland. We recruited adult patients attending renal clinics in the West of Scotland with a diagnosis of either diabetic nephropathy or biopsy-proven IgA nephropathy and CKD stages 3-4. Patients with essential hypertension in the absence of CKD were recruited from the Glasgow blood pressure clinic. Patients who were receiving immunosuppression, had evidence of active infection, or who were unable to undergo CMRI scanning were excluded. Written informed consent was obtained from all patients and the study was approved by the local ethics committee.

Patients attended the Glasgow Clinical Research Facility. Demographic data including height, weight, and waist circumference were recorded. The lowest of three blood pressure measurements was recorded. Investigations included measures of renal function (estimated by the Modification of Diet in Renal Disease (MDRD-4) formula), bone biochemistry (PTH, vitamin D, calcium, and phosphate levels), haemoglobin and C-reactive protein (CRP). Proteinuria was estimated from a spot protein : creatinine ratio (PCR), and 24-h proteinuria quantification (24 h QP). All prescribed medications were continued throughout the study.

2.2. FGF-23 Measurement. FGF-23 concentrations were measured on EDTA plasma samples frozen and stored at −80°C. Samples were measured in duplicate after a single thaw, according to the manufacturer's instructions, using the 2nd generation Human C-terminal FGF-23 ELISA (Immunotopics Inc., San Clemente, CA, USA). Recombinant human FGF-23 was used as standard at concentrations of 0.18.50.150.445 and 1500 RU/mL. The sensitivity of the assay is 1.5 RU/mL.

2.3. Cell-Based ELISA. HUVECs from pooled donors were obtained from PromoCell, Heidelberg, Germany. The cells were seeded into tissue culture treated 75 mL flasks (Corning incorporated, NY, USA) and grown in either standard endothelial cell basal medium which has a phosphate concentration of 0.5 mM or in custom formulated media which has a phosphate concentration of 3 mM. Both media were obtained from PromoCell and supplemented with the endothelial cell growth medium 2 supplement mix and 100 mg/mL penicillin/streptomycin. All cultures were incubated at 37°C in 5% CO_2. The cells were used between the 2nd and 4th passage. 18,000 cells per well were added to 96 well plates. The cells were left for 24 hours and then treated with FGF-23 ($3.5e^{-7}$) and Klotho ($2e^{-10}$) either alone or in combination; controls were untreated. After four hours incubation with FGF-23 and Klotho, some wells were also treated with Interleukin 1β (IL1), and all plates left for a further six hours. The wells were washed with phosphate buffered saline (PBS) and then the cells were fixed with paraformaldehyde (4% dissolved in a 5% sucrose solution) and incubated at 4°C overnight. The wells were washed with a PBS and 0.1% BSA solution for one hour to block nonspecific protein binding. For E-selectin, mouse antihuman antibody (AbD Serotec, Oxford, UK) at a 1/2000 dilution was added to each well. For VCAM sheep antihuman antibody (R&D Systems, Oxford, UK) at a 1/2000 dilution was added to each well. After a one-hour incubation, the wells were washed and the secondary antibody added (horseradish peroxidase coupled with mouse for E-Selectin and with goat for VCAM, both from Sigma-Aldrich, MO, USA). This was washed off after incubation for one hour and developer was added (TMB from Sigma-Aldrich). The wells were read at 630 absorbance on a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). Stripping buffer was added to the wells which were then probed for mouse anti-human GAPDH. Cell-based ELISA for E-Selectin and VCAM was performed in 3 separate experiments with each group represented in triplicate in each experiment and results were normalized to GAPDH.

2.4. Cardiac Magnetic Resonance Imaging Scanning. Our CMRI protocol has previously been described [22, 23]. Briefly, noncontrast CMRI was performed to determine left ventricular mass index (LVMI) using 8 mm thick short-axis cine slices from a 1.5-Tesla Siemens (Erlangan) CMRI scanner, and a fast imaging with a steady-state precision (FISP) sequence. LVM was analysed by a blinded observer from short-axis cine loops using manual tracing of epicardial and endocardial end-systolic and end-diastolic contours. End-systolic volume (ESV), end-diastolic volume (EDV), and LVM were calculated using commercial software (Argus; Siemens). Values were adjusted for body surface area (Mosteller formula, BSA (m^2) = $\sqrt{((\text{weight (kg)} \times \text{height (cm)})/3600)}$, and LVH was defined as LVMI > 84.1 g/m^2 for men and >76.4 g/m^2 for women; LV systolic dysfunction was defined as LV ejection fraction (LVEF) <55% [24].

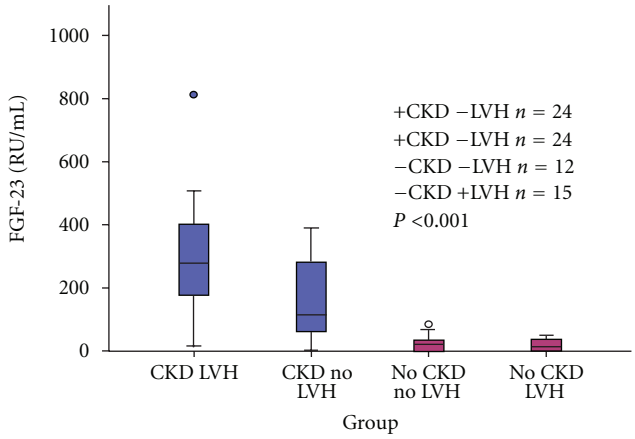

Figure 1: FGF-23 concentration stratified according to the presence or absence of CKD and LVH.

2.5. Statistical Analyses. Statistical analysis was carried out using the IBM SPSS statistics package version 18.0 (IL, USA). Normality of the data was determined using Kolmogorov-Smirnoff analysis with normally distributed data expressed as mean and SD and nonnormally distributed data as median and interquartile range (IQR). Parametric correlations were calculated using Pearson's correlation, nonparametric using Spearman's. Variables were log-transformed to obtain a normal distribution as required. Comparisons were made using Student's t-test, Mann Whitney U test, and one way analysis of variance as appropriate. Multivariate analysis was performed using binary logistic regression to determine independent predictors of LVH.

For cell-based ELISA comparisons, the different groups were compared using a two-way analysis of variance and *post hoc* analysis was carried out with Tukey's test.

3. Results

48 patients with CKD were included in the analysis. 62% ($n = 36$) had a diagnosis of diabetic nephropathy, and 18% had IgA nephropathy. 50% ($n = 24$) had LVH. 27 "control" patients with essential hypertension but without CKD were also included in the analysis; 56% ($n = 15$) of them had LVH. Comparison between the patients with and without CKD is shown in Table 1. There was a similar age distribution (60 ± 12 years versus 55.5 ± 9.5 years) and a similar proportion of males (75% versus 74%). LVMI was also similar between the two groups (81.2 g/m^2 IQR70.2-97.6 versus 86 g/m^2 IQR 71.6-99.6). Patients with CKD had significantly higher phosphate, parathyroid hormone, and FGF-23 concentration (237.9 RU/mL IQR 109.6-393.3 versus 12.5 RU/mL IQR 1.5-35.9, $P < 0.001$).

Figure 1 shows stratification of patients by median FGF-23 concentration into 4 groups according to the presence or absence of LVH and CKD. Those with both CKD and LVH had the highest FGF-23 concentration followed by those with CKD without LVH (283.4 RU/mL IQR 166.4-414.8 versus 118.4 RU/mL IQR 61.5–295, $P < 0.001$).

Table 1: Comparison of the baseline characteristics between the patients with CKD and control patients with essential hypertension (EH).

	CKD ($n = 48$)	EH ($n = 27$)	P value
Age (years)	60 ± 12	55.5 ± 9.5	0.072
Male sex	75% ($n = 36$)	74% ($n = 20$)	0.94
DM nephropathy	62% ($n = 30$)	NA	
eGFR (mL/min/1.73 m^2)	30.2 ± 11	94.4 ± 11.1	<0.001
LVMI	81.2 (70.2–97.6)	86 (71.6–99.6)	0.419
Systolic BP (mmHg)	148.8 ± 22.9	152.29 ± 19.9	0.48
Diastolic BP	81.8 ± 12	94.4 ± 11.1	<0.001
Phosphate (mmol/L)	1.2 ± 0.2	0.99 ± 0..22	<0.001
Vitamin D (ng/mL)	13 (5–20)	20.5 (14–26.8)	0.004
FGF-23 (RU/mL)	237.9 (109.6–393.3)	12.5 (1.5–35.9)	<0.001
PTH (pg/mL)	14.5 (6.7–21.6)	5.7 (4.8–7.3)	<0.001
Calcium (mmol/L)	2.38 ± 0.1	2.4 ± 0.07	0.096
Ur PRC (mg/mmol)	82.5 (19.75–218)	NA	

A scatter plot of FGF-23 concentration and LVMI in patients with CKD is shown in Figure 2 demonstrating significant positive correlation between the 2 ($r = 0.064$, $P = 0.005$). Figure 3 shows the negative correlation with FGF-23 concentration and renal function ($r = 0.072$, $P = 0.004$). There was also a positive correlation with phosphate ($r = 0.073$, $P = 0.006$) (data not shown). Further comparison between the patients with CKD with and without LVH is shown in Table 2. eGFR was significantly lower in those with LVH (27.5 mls/min ± 11.7 versus 34.3 mls/min ± 11.3 $P = 0.044$) and systolic blood pressure and PCR significantly higher (158.8 ± 19.7 versus 139.2 ± 22.2,$P = 0.002$ and 82.5 IQR 19.8–218 versus 67.4 IQR 12.5–196, $P = 0.004$). Multivariate analysis including creatinine, FGF-23, systolic blood pressure, and urinary PCR revealed all, except creatinine, to be independent predictors of LVH (Table 3). Creatinine was used rather than eGFR calculated by MDRD 4 because LVH already takes into account body mass and it was felt that it was more appropriate to use creatinine. We did try the model with eGFR and this was not a significant determinant of LVH.

In the second part of our study we looked at the effects of altered phosphate concentration in the presence and absence of FGF-23, Klotho and IL1 on the production of the cell adhesion molecules E-selectin and VCAM. Compared with cells stimulated only with IL1, both E-selectin and VCAM production is significantly elevated in HUVECs cultured in high-phosphate media (3 mM) in the presence of FGF-23 and IL1 or Klotho and IL1 ($P = 0.005$ and 0.035). This effect is attenuated in the cells exposed to both FGF-23 and Klotho with IL1. Figure 4 shows representative experiment results for E-selectin production from one experiment performed in

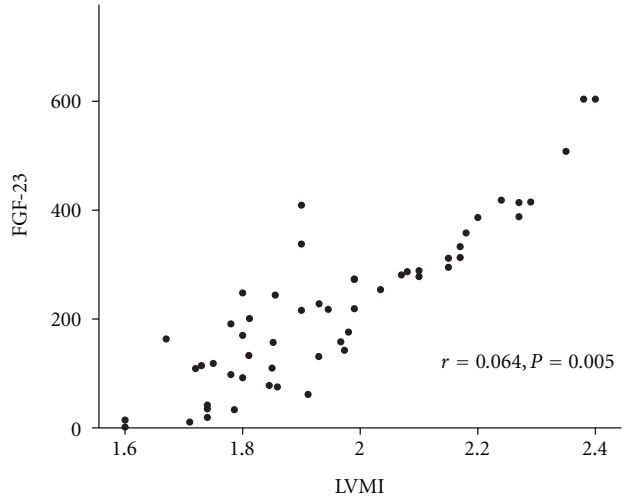

Figure 2: Scatter plot showing the correlation between FGF-23 concentration and LVMI in patients with CKD.

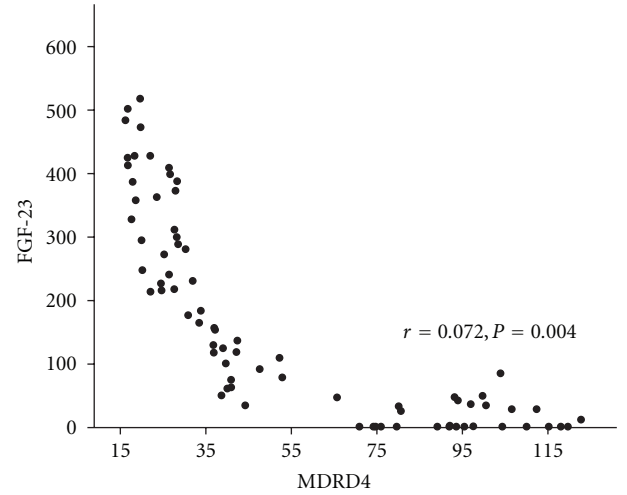

Figure 3: Scatter plot showing the correlation between FGF-23 concentration and renal function by the MDRD4 equation in all patients.

triplicate. There was no significant difference seen between cells which were not stimulated with IL1.

4. Discussion

In this study, we have looked at the associations of FGF-23 and vascular structure in CKD, with additional in vitro studies on the underlying mechanism. It is the first study to assess the relationship between FGF-23 and LVH measured with CMRI. We confirm the findings of other studies which have used less sensitive measures of LVH; FGF-23 level is an independent predictor of LVH in patients with CKD 3 and 4 secondary to diabetic or IgA nephropathy.

Patients without CKD had much lower levels of FGF-23 regardless of the presence or absence of LVH (13.8 RU/mL IQR 1.5–38 versus 20.7 RU/mL IQR 1.5–42.2 $P = 0.43$).

Table 2: Comparison between patients with CKD with and without LVH.

	LVH ($n = 24$)	No LVH ($n = 24$)	P value
Age (years)	60.4 ± 11.6	58.8 ± 12.8	0.652
Male sex	75% ($n = 18$)	71% ($n = 17$)	0.54
DM nephropathy	67% ($n = 16$)	58% ($n = 14$)	0.34
eGFR (mL/min/1.73 m^2)	27.5 ± 11.7	34.3 ± 11.3	0.044
LVMI	87.9 (84.3–95.1)	79.6 (73.2–83.8)	0.045
Systolic BP (mmHg)	158.8 ± 19.7	139.2 ± 22.2	0.002
Phosphate (mmol/L)	1.2 ± 0.19	1.16 ± 0.22	0.21
Vitamin D (ng/mL)	13 (5–20)	14 (4–22)	0.94
FGF-23 (RU/mL)	283.4 (166.4–414.8)	118.4 (61.5–295)	0.008
PTH (pg/mL)	14.5 (6.7–21.6)	13.1 (5.9–19.6)	0.25
Calcium (mmol/L)	2.34 ± 0.06	2.37 ± 0.07	0.9
Ur PCR (mg/mmol)	82.5 (19.8–218)	67.4 (12.5–196)	0.004

Table 3: Multivariate analysis model showing significant predictors of LVH in patients with CKD.

	Exp β	Confidence interval		P value
		Lower	Upper	
FGF-23	4.9	1.2	20.3	0.027
Systolic BP	1.08	1.03	1.14	0.003
Ur PCR	1.87	1.54	2.23	0.005

Left ventricular wall thickness increases as the heart adapts to chronic pressure overload with resultant LVH [25]. LVH is found in up to 25% of essential hypertensives without end-organ damage elsewhere, and up to 60% in those with end-organ damage in other territories [26]. The low levels of FGF-23 seen in our essential hypertension group with LVH suggest that FGF-23 is neither a biomarker for LVH nor does it contribute significantly to the development of LVH in patients without CKD.

LVH is present in up to 75% of patients starting dialysis. Bregman et al. studied factors affecting LVH in patients with CKD 3 and 4. They demonstrated that it affects approximately 50% of patients with this level of CKD and is more common with more advanced CKD [27]. Both systolic and diastolic blood pressures are described in the literature as independent predictors of LVH in patients with and without CKD [28, 29]. There is an increased prevalence of LVH in patients with CKD and it may develop after shorter exposure to hypertension and possibly at lower values. In animal models, LVH develops in CKD even without significant hypertension [30]. This supports the argument that additional factors contribute to the development of LVH. There are many other factors that may be associated

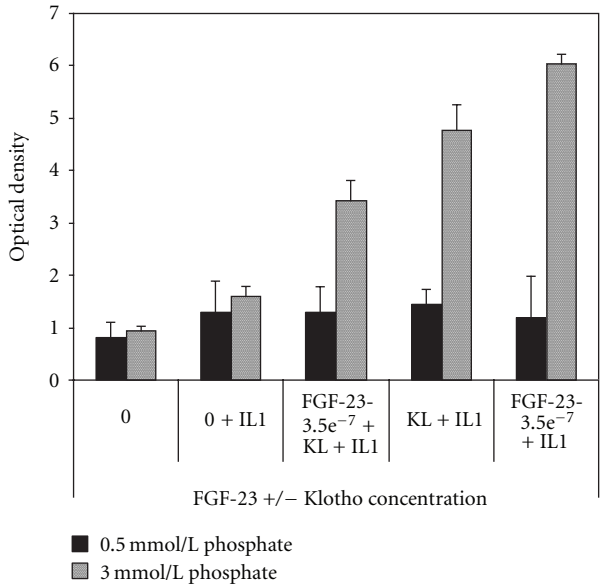

Figure 4: E-selectin production in HUVECs grown in high (3 mM) and normal (0.5 mM) phosphate concentration media and stimulated with FGF-23, klotho, and IL1.

with LVH in CKD including age, anaemia, proteinuria, and FGF-23 level [9, 22, 26, 31]. In our studies of patients with stage 5 CKD, blood pressure is the major determinant of LV mass. However calcium-phosphate product is an additional contributor to LVH in keeping with the hypothesis that CKD mineral bone disease dysregulation is implicated in the pathogenesis of LVH in CKD [23]. LVH is a major predictor of cardiovascular mortality in advanced CKD and potentially a risk factor for progression to dialysis [32]. Regression of LVH therefore represents a possible strategy for improving cardiovascular outcomes in CKD patients.

In the current study, we show that FGF-23 level is an independent predictor of LVH in CKD and show the levels to be significantly higher than in those with CKD who do not have LVH. Since patients with CKD develop LVH at lower blood pressure levels than patients without CKD, some other factor present in CKD is likely to be involved. Our data support the notion that FGF-23 may be responsible.

To support a pathophysiological role for FGF-23, it is necessary to show that FGF-23 either has direct effects on the growth or matrix production of cardiac myocytes (or other vascular cells) or that it has the potential to increase blood pressure. We examined the effect of FGF-23 on endothelial cells and show that FGF-23 stimulates the production of the cell adhesion molecules, E-selectin and VCAM. Higher levels of E-selectin and VCAM, indicate activation of the vascular endothelium and are present in patients with essential hypertension patients, who have endothelial dysfunction [20]. In the presence of Klotho, this effect is attenuated consistent with the established role of Klotho as an antiaging protein [33]. Thus, FGF-23 may cause activation and dysfunction of the vascular endothelium and contribute to hypertension and LVH in patients with CKD. This is a small pilot study and our cell-based ELISA findings require confirmation with Western blot. Additionally, gene expression of E-selectin and VCAM requires evaluation in cells treated with phosphate, FGF-23, and Klotho.

It remains to be shown whether there are similar direct effects on cardiac myocytes, but our findings support the hypothesis that FGF-23 is more than a biomarker of abnormal renal phosphate handling and may be a therapeutic target as phosphate-binding medication reduces FGF-23 levels [34].

5. Conclusion

In this small, single centre, pilot study, we have show that FGF-23 is associated with the development of LVH in CKD, as measured by the "gold standard" method of CMRI and provide preliminary evidence of a direct effect in vitro that may contribute to endothelial dysfunction and LVH. We do not prove causality with elevated FGF-23 and LVH and further research is necessary to ascertain if lowering levels of FGF-23 offers benefit in terms of improved clinical outcomes.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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