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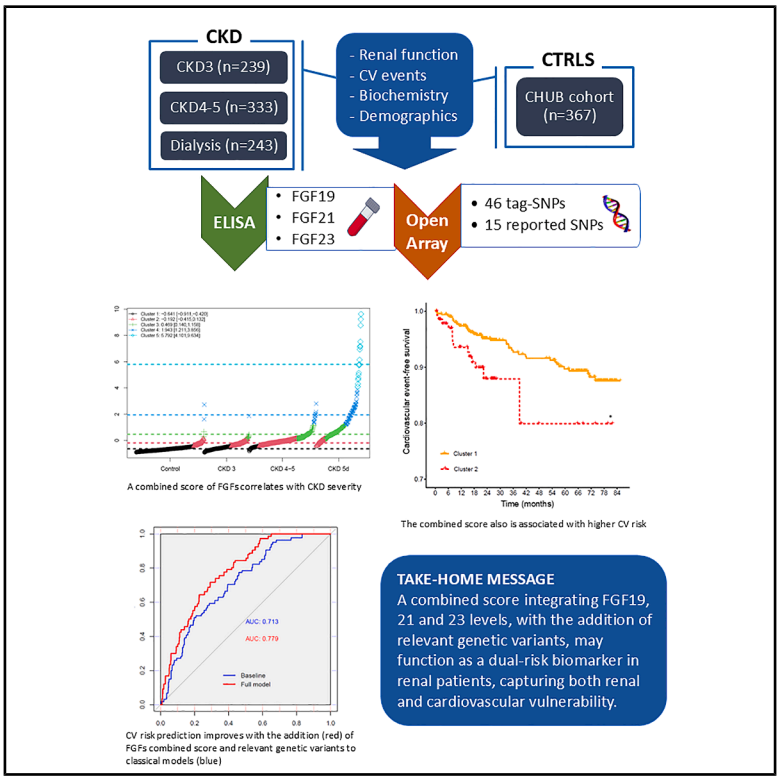
Combined concentrations and genetic variability of fibroblast growth factors predict cardiovascular risk in renal patients

Graphical abstract



Highlights

- Combined FGF19/21/23 levels rise with CKD severity and CV risk
- FGF19, FGF23, and FGFR4 SNPs link to CV outcomes in CKD
- FGF biomarker-genetic models improve CV risk prediction beyond classics
- Integrating FGF data may personalize cardiorenal risk management

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In brief

Health sciences

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Combined concentrations and genetic variability of fibroblast growth factors predict cardiovascular risk in renal patients

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SUMMARY

Chronic kidney disease (CKD) is a major risk factor for cardiovascular events (CVE). We assessed whether circulating levels and genetic variability of endocrine fibroblast growth factors (FGF19, FGF21, and FGF23) could predict CV risk in these patients. In 1,182 participants (815 CKD patients and 367 controls), plasma FGF concentrations and 46 gene variants were analyzed, with participants followed-up for a mean of 37.6 ± 25.7 months for CVE. Clustering based on combined scores for all three FGF concentrations correlated strongly with CKD severity ($p < 0.001$) and predicted CVE after adjusting for other risk factors [hazard ratio (HR) = 2.03 (1.02–4.05), $p = 0.044$]. Four SNPs, notably FGF19 rs1307968 [odds ratio OR = 5.14 (1.53,17.27), $p = 0.008$], were also independently associated with CVE. Incorporating both combined FGF concentration scores and the relevant genetic variants into traditional risk models significantly improved prediction accuracy (AUC increased from 0.713 to 0.779; $p < 0.0001$). These findings suggest that combining FGF biomarkers with genetic information may enhance CV risk stratification in CKD patients.

INTRODUCTION

The cause of chronic kidney disease (CKD) is unknown in a significant percentage of patients, which hinders an etiological diagnosis, targeted therapy, and early prevention campaigns. Additionally, traditional markers of the disease such as glomerular filtration rate (GFR) estimation and albuminuria only stand out when kidney damage is advanced, but early identification of CKD is important to improve patients' survival and reduce associated comorbidities, particularly of cardiovascular (CV) nature, hence the need for novel biomarkers.[1]

The fibroblast growth factor (FGF) family comprises 22 members, of which three (FGF19, FGF21, and FGF23) act as circulating hormones.[2] Alterations in circulating concentrations of these endocrine FGFs have been implicated in the pathogenesis of multiple chronic diseases and have recently been pointed out as potential biomarkers of CKD.[3,4] FGF19 is a regulator of bile acid synthesis and glucose metabolism, which has been associated with diabetes and CV risk.[5,6] However, studies on this factor in CKD are very scarce, with small sample sizes and conflicting findings.[4,7–9] With regard to FGF21, elevated concentrations have been linked to CV disease (CVD) in the general population[10] and to abnormalities in glucose and lipid metabolism in end-stage kidney disease (ESKD), again mostly in undersized studies.[8,11] Finally, FGF23, whose major target is the kidney, induces urinary phosphate excretion.[12] Circulating levels of FGF23 increase early during CKD and may increase up to 1,000-fold in patients on dialysis.[13,14] Furthermore, its effect on poor outcomes in CKD may be modulated by its impact on anemia and systemic inflammation.[15]

Genetics also plays a role in CKD; however, and somewhat surprisingly given the aforementioned background, data on the

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impact of genetic variants in these genes are very scarce in the renal setting. There are some interesting results regarding their association with renal function[16] or CV events (CVEs),[17,18] but results with regard to CKD specifically have been controversial.[19] In general, available studies only analyze a small number of known SNPs, mainly in the *FGF23* gene, while *FGF21* or *FGF19* variability remains virtually unexplored.

In the present work, we have measured plasma concentrations of FGF19, 21, and 23 in a large group of patients with CKD and ESKD and control subjects, as well as identified clinically relevant genetic variants and tag-SNPs. These are variants that capture the whole genetic variability of *FGF19*, *FGF21*, and *FGF23* and their respective receptors (*FGFR1* and *FGFR4*). With this, the primary goal of this study was to develop a model containing FGF concentrations and related genetic variants, which, in conjunction with other relevant clinical and demographic characteristics, may be useful to predict CV risk in CKD patients. Secondarily, we also aimed to establish whether the combined study of endocrine FGFs may have a prognostic value in CKD, as well as to determine the effect of genetic variability on the circulating concentrations of these FGFs.

RESULTS

Median age and interquartile range (IQR) were 68 years (17) for CKD patients and 71 years (17) for controls, while the percentage of male subjects was 64.4% and 51.9% in these two groups. The prevalence of classical CV risk factors, including diabetes mellitus, hypertension, smoking, and dyslipidemia, was significantly elevated in the CKD group ($p < 0.0001$ in all cases). As anticipated, biochemical parameters also differed greatly between CKD patients and controls. Among the most frequent causes for CKD, diabetic nephropathy ranked first (24.5%), followed by nephroangiosclerosis (17.9%) and interstitial nephropathy (11.7%). Causes were unknown in 18.2% of cases. Table 1 summarizes demographic and clinical characteristics of the study population.

Endocrine FGF plasma levels

Plasma concentrations of all assessed endocrine FGFs significantly differed between CKD stages (Figure S1). Median (IQR) values for controls, CKD3, CKD4-5, and CKD5d were as follows: for FGF19: 124.5 (129.43), 153.5 (138.65), 257.0 (182.0), and 357.0 (350.0) pg/mL, $p < 0.0001$; for FGF21: 140.5 (256.58), 352.0 (504.0), 708.0 (914.0), and 1,267.0 (2,090.0) pg/mL, $p < 0.0001$; and for FGF23: 88.9 (138.4), 202.0 (204.5), 623.0 (591.0), and 1,971.0 (3,464.0) pg/mL, $p < 0.0001$.

A combined score for the three FGF levels was obtained for each participant using exploratory factor analysis (see detailed description in STAR Methods), with a Cronbach's alpha of 0.77. The score was computed summarizing the standardized values of FGF19, FGF21, and FGF23 weighted by 0.282, 0.461, and 0.799, respectively. Subjects were then assigned to five different groups according to a cluster analysis based on this score. Figure 1 shows that individuals with higher scores were predominantly found in the most severe stages of the disease. Conversely, lower scores were massively overrepresented in subjects with higher eGFR values. Differences were statistically significant between all CKD stages ($p < 0.001$).

Given that these endocrine FGFs have also been involved in glucose homeostasis, insulin resistance, and diabetes, we also assessed whether the FGF combined score was associated with the incidence of diabetic nephropathy, the most common cause of CKD in our cohort. This association was not statistically significant [median and IQR scores for patients with diabetic nephropathy or other causes were −0.03 (1.01) and −0.07 (0.80), $p = 0.815$, respectively]. However, when we analyzed the association with diabetes, diabetic patients had significantly higher scores than nondiabetic patients [−0.14 (0.85) vs. −0.43 (0.67), $p < 0.001$]. The fact that many diabetic patients had their CKD cause diagnosed as "unknown" instead of "diabetic nephropathy" is most likely behind this observation.

Impact of genetic variants on circulating levels of endocrine FGFs

A total of 787 participants consented to SNP genotyping. We analyzed the influence of variants in the five genes studied (*FGF19*, *FGF21*, *FGF23*, *FGFR1*, and *FGFR4*) on the circulating endocrine FGFs levels. A linear regression model adjusted for age, sex, body mass index (BMI), hypertension, diabetes, and smoking was performed in a codominant model of inheritance. Figure 2 shows the degree of statistical association for the assessed SNPs. Remarkably, six (rs2231861, rs2548957, rs35650232, rs739320, rs838133, and rs499765) of the eleven *FGF21* SNPs significantly modified FGF19 concentrations. After Bonferroni correction for multiple testing, carriers of the homozygous variant genotypes of rs739320 and rs838133 still showed reduced FGF19 levels compared with wild-type carriers (215.94 ± 137.23 vs. 306.55 ± 222.66 pg/mL, $p < 0.001$ and 233.23 ± 170.67 vs. 324.43 ± 238.44 pg/mL, $p < 0.001$, respectively). *FGF21* rs838133 was also significantly associated with FGF23 plasma levels (992.6 ± 1868.0 vs. 1815.6 ± 4597.8 pg/mL, $p < 0.05$); however, this association did not survive Bonferroni correction. Detailed information on all selected SNPs can be found in Table S1.

Association of endocrine FGF levels with cardiovascular event-free survival

A total of 836 participants were followed-up for a mean of 37.6 ± 25.7 months to record the occurrence of CVE. Sixty-nine events (8.3%) were registered in this period, the vast majority of them (64) in the CKD patients. Subjects who experienced CVEs were older ($p < 0.01$), had higher BMI ($p < 0.05$) and potassium values ($p < 0.05$), and showed a higher incidence of diabetes ($p < 0.0001$) and greater occurrence of CVE prior to the start of the study ($p < 0.01$). These and other features of individuals with and without CVE are listed in Table 2.

Kaplan-Meier analyses showed that higher levels of FGF21 and FGF23 were associated with worse event-free survival (log rank $p = 0.035$ and $p < 0.0001$, respectively). Figure 3 shows the analysis by tertiles (T) conducted with Cox models adjusted for other risk factors, namely age, sex, BMI, hypertension, diabetes, total cholesterol, calcium levels, smoking, estimated GFR (eGFR), and CKD diagnosis, where higher FGF23 concentrations were associated with worse event-free survival: T1 vs. T3 [22.00 ± 12.42 vs. 62.14 ± 22.07 months, hazard ratio (HR) = 2.96 (1.12–7.86), $p = 0.029$]. In addition, a statistical

Table 1. Characteristics of the participants included in the study

	Control (*n* = 367)	CDK3 (*n* = 239)	CDK4-5 (*n* = 333)	CDK 5D (*n* = 243)	p^a	p^b
Males (%)	190 (51.8%)	157 (65.7%)	215 (64.6%)	153 (63.0%)	<0.0001	0.820
Age (years)	68.0 (17.0)	72.0 (14.0)	71.0 (19.0)	70.0 (19.0)	<0.0001	0.734
Weight (kg)	82.0 (21.88)	79.0 (15.6)	78.85 (21.43)	72.65 (18.25)	0.044	<0.0001
BMI	28.38 (5.92)	29.05 (5.69)	28.80 (7.22)	26.60 (8.63)	0.003	<0.0001
Glucose (mg/dL)	100.5 (20.0)	107.0 (46.0)	101.5 (30.0)	115.0 (69.0)	0.006	<0.0001
Total cholesterol (mg/dL)	174.0 (44.0)	171.0 (59.0)	144.0 (48.0)	139.0 (45.0)	<0.0001	<0.0001
Cholesterol HDL (mg/dL)	54.5 (21.0)	46.0 (19.0)	44.0 (20.0)	39.0 (20.0)	<0.0001	0.001
Cholesterol LDL (mg/dL)	96.0 (36.0)	91.0 (49.0)	68.7 (40.0)	67.0 (36.0)	<0.0001	<0.0001
Dyslipidemia						
Yes	56 (31.5%)	51 (55.4%)	229 (70.9%)	122 (50.4%)	<0.0001	<0.0001
No	122 (68.5%)	41 (44.6%)	93 (28.8%)	120 (49.6%)		
Hemoglobin	14.45 (1.9)	13.6 (2.35)	11.7 (1.92)	11.2 (1.9)	<0.0001	<0.0001
Calcium (mg/dL)	9.45 (0.4)	9.55 (0.61)	9.3 (0.7)	9.25 (0.68)	<0.0001	<0.0001
Potassium (mEq/L)	4.4 (0.5)	4.7 (0.7)	4.9 (0.8)	4.75 (1.15)	<0.0001	<0.0001
Sodium (mEq/L)	141.0 (3.0)	142.0 (3.0)	141.0 (3.0)	140.0 (6.0)	<0.0001	<0.0001
Phosphorus (mg/dL)	3.2 (0.79)	3.4 (0.71)	3.9 (1)	4.2 (1.4)	<0.0001	<0.0001
ACR (mg/g)	8.52 (27.43)	82.97 (267.69)	410.19 (1052.51)	–	<0.0001	<0.0001
eGFR (ml/min/1.73 m^2)	99.0 (22.35)	41.83 (17.19)	17.0 (7.0)	–	<0.0001	<0.0001
Albuminuria (mg/24 h)	10.85 (34.61)	105.12 (395.93)	420.0 (1070.53)		<0.0001	<0.0001
Cystatin C	0.85 (0.28)	1.52 (0.57)	2.81 (0.91)	–	<0.0001	<0.0001
PTH	55.97 (30.26)	66.25 (49.43)	182 (169.5)	315 (282)	<0.0001	<0.0001
Vitamin D	–	22.6 (16.96)	21.6 (14.4)	24.4 (13.72)	–	0.325
C-reactive protein (mg/L)	1.4 (1.95)	2.65 (7.31)	2.9 (6.25)	3.9 (8)	0.0003	0.129
Hypertension						
Yes	218 (59.6%)	197 (82.4%)	286 (85.9%)	180 (75.3%)	<0.0001	0.005
No	148 (40.4%)	42 (17.6%)	47 (14.1%)	59 (24.7%)		
DM						
Yes	62 (16.9%)	94 (39.3%)	158 (47.4%)	126 (52.7%)	<0.0001	0.013
No	305 (83.1%)	144 (60.7%)	175 (52.6%)	113 (47.3%)		
HbA1c (%)	6.3 (1.35)	6.8 (1.67)	6.6 (1.3)	6 (1.25)	0.043	0.0005
Smoking						
Non-smoker	215 (59.9%)	102 (43.6%)	148 (44.6%)	115 (51.09%)	<0.0001	0.278
Former smoker	89 (24.8%)	91 (38.9%)	137 (41.3%)	83 (36.7%)		
Current smoker	55 (15.3%)	37 (15.8%)	47 (14.2%)	28 (12.4%)		
Systolic pressure (mmHg)	132.0 (24.0)	140.0 (27.0)	143.0 (35.0)	141.0 (31.0)	<0.0001	0.076
Diastolic pressure (mmHg)	80.0 (16.0)	78.0 (17.0)	74.0 (19.0)	64.0 (20.0)	<0.0001	<0.0001
Pulse pressure (mmHg)	52.0 (21.0)	61.0 (25.0)	68.0 (34.0)	70.5.0 (30.0)	<0.0001	0.004

BMI, body mass index; eGFR, estimated glomerular filtration rate; CVE, cardiovascular event; DM, diabetes mellitus; ACR, albumin to creatinine ratio.
Median (interquartile range) or count (percentage) values are shown.
[a]*p* values for controls vs. all CKD patients.
[b]*p* values for the difference between CKD stages.

trend toward higher CV risk was observed for individuals in the T1 of FGF19 concentrations compared to those in T3 [27.70 ± 20.79 vs. 47.74 ± 26.87 months, HR = 1.73 (0.86–3.47), $p = 0.123$].

Next, subjects were divided into two groups according to the cluster analysis based on the combined score for all three FGFs concentrations. Figure 4 shows that individuals within cluster 2, with higher scores, had significantly lower CV event-free survival compared to those in cluster 1 according to the adjusted Cox model [21.46 ± 14.73 vs. 40.53 ± 26.24 months; HR = 2.03 (1.02–4.05), $p = 0.044$].

Association of genetic variability in endocrine FGF genes with cardiovascular events

Four out of the 46 variants studied were found to be significantly associated with CV risk in Cox regression models

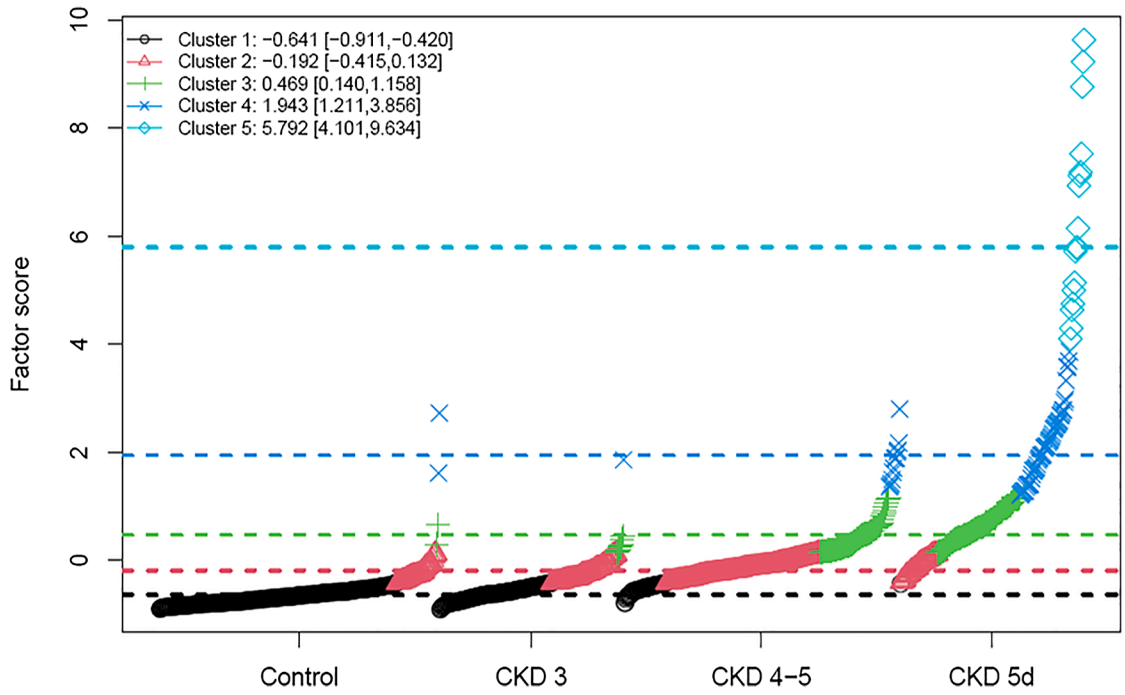

Figure 1. Distribution of combined scores summarizing plasma levels of FGF19, FGF21 and FGF23 in the participants according to the severity of chronic kidney disease

CKD5d stage denotes patients on dialysis. CKD, chronic kidney disease. Median [minimum, maximum] values are given for each cluster.

adjusted by age, sex, BMI, diabetes, hypertension, smoking status, history of CVE, and CKD diagnosis. Table 3 shows that a gene-dose effect was observed for *FGF19* rs1307968, as HR values increased from 1.84 (1.01,3.36), $p = 0.048$ for heterozygous carriers to 5.14 (1.53,17.27), $p = 0.008$ for homozygous carriers. In addition, homozygous variant genotypes of *FGF23* rs11063112 ($p = 0.006$) and *FGFR4* rs31776 ($p = 0.003$) also significantly increased the risk for CVE. In contrast, heterozygous carriers of *FGFR4* rs351855 displayed better CV event-free survival ($p = 0.024$).

Risk model for cardiovascular events

Finally, we assessed the putative role of the relevant genetic variants (rs1307968, rs11063112, rs31776, and rs351855) and the two-group cluster based on the combined score representing FGFs levels as CV biomarkers by using ROC analysis. The addition of these two elements to a multivariate logistic regression model consisting of classic risk CV factors significantly increased its area under curve (AUC) from 0.713 (0.686,0.739) to 0.779 (0.756,0.802), $p < 0.0001$ (Figure 5).

DISCUSSION

CKD remains highly prevalent and is strongly associated with increased risk of adverse CV outcomes.[20] Effective CKD prevention depends on early risk identification and strategies to slow progression, but these efforts are hindered by limited understanding of its etiopathogenesis, highlighting the need for studies to identify predictive biomarkers.

In the present work, plasma levels of all endocrine FGFs studied showed a consistent correlation with the severity of CKD. The relationship between FGF23 and CKD has long been known.[13] In contrast, fewer studies, with limited sample size and some conflicting results, have analyzed FGF21, particularly FGF19 in this setting.[4,7–9] Our findings not only confirm that all endocrine FGF levels are dysregulated in CKD but also highlight the potential utility in clinical practice of a composite FGF score integrating circulating concentrations of FGF19, FGF21, and FGF23. By capturing multisystemic biochemical alterations associated with mineral metabolism, metabolic stress, and inflammation, the score utilized in the present work may provide a more nuanced and biologically relevant stratification than that obtained from the levels of individual FGFs, as well as open the door to the use of additional biomarkers of CKD severity beyond traditional parameters like eGFR and albuminuria. In the same line, Toro et al. have reported the use of a combined biomarker, including FGF23 and other non-FGF compounds, for the development of CKD in patients with sepsis.[21]

Given the interconnection of endocrine FGFs with pathways perturbed in CKD and implicated in CVD, these factors have garnered significant attention as potential biomarkers for CV risk in renal patients.[2,22] Our findings showed that patients with elevated FGF23 levels had lower CV event-free survival after adjustment for other risk factors. This agrees with previous studies that demonstrated an independent association between high FGF23 levels and increased risk of CVE in CKD patients (reviewed in[23–25]), an association that appears to be more pronounced in individuals with CKD compared to those without the condition.[26]

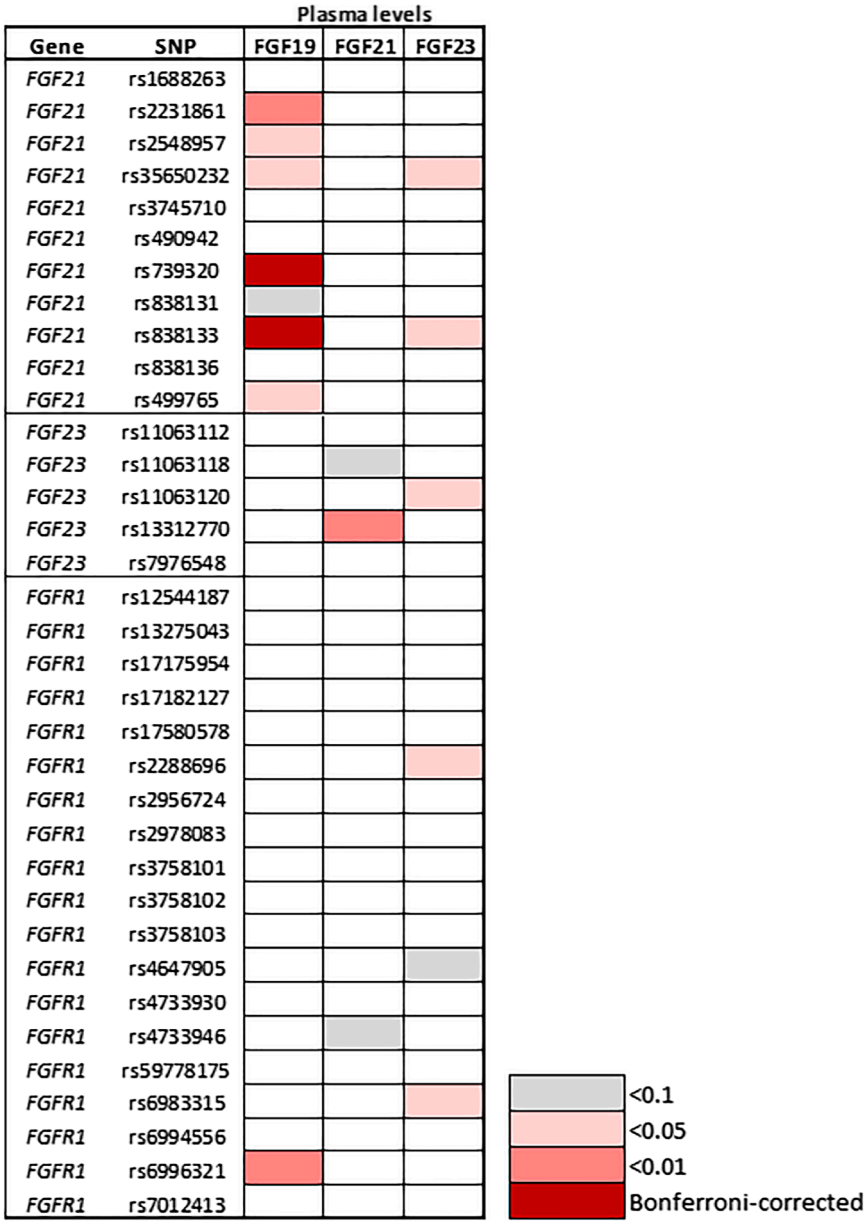

Figure 2. Influence of genetic variants on endocrine FGFs plasma concentrations in a codominant model of inheritance

Variants in *FGF19* and *FGFR4* genes are not shown as they did not produce any significant association. Darker colors denote a higher degree of association.

Numerous physiological explanations, e.g., endothelial dysfunction or arterial stiffness, have been proposed for the impact of raised FGF23 on CVD,[27–31] but it should be remarked that it is still debatable whether FGF23 levels follow, rather than induce, CVD in conditions such as CKD.[23] In addition, we observed a statistical trend indicating that higher levels of FGF19 were also associated with lower event-free survival rates. In this regard, available studies for FGF19 are not only scarce but also contradicting. Hao et al. have reported that low FGF19 levels were associated with increased severity of coronary artery disease.[32] Conversely, a positive correlation between FGF19 concentrations and subclinical atherosclerosis in male diabetic patients has also been found,[33] while Yamamoto et al. described that FGF19 levels tended to be high in older CKD patients with a history of CVD,[4] reports that agree with our results. It should be noted, however, that the overall existing evidence of the involvement of FGF19 in CVD is often times indirect and, in any case, considerably weaker than that of FGF23. Finally, regarding FGF21, its association with CVD lost significance when adjusted by other risk factors. In the same line, a very recent mendelian randomization study by He et al., which aimed to establish a causal relationship between FGF21 and six CVDs, found no evidence of this link.[34] In any case, studies suggesting the opposite also exist,[35,36] although none of them was carried out in the CKD setting. Most interestingly, we have shown that the calculated FGF combined score not only correlated with CKD severity, but it was also an independent CV risk factor, which suggests that this tool could play a role in improving the integrated care of CKD patients, particularly in relation to CV risk, a major concern in this population.

Considering the importance of endocrine FGF levels for CKD, it is undoubtedly relevant to analyze intrinsic factors of the patient that could alter these concentrations. A remarkable finding was that several *FGF21* genetic variants, particularly rs739320 and rs838133, were associated with modified FGF19 plasma levels. Given that FGF21 and FGF19 participate in overlapping regulatory pathways—particularly those governing the regulation of energy balance and the metabolism of glucose, bile acid, and lipids—the presence of genetic variants influencing FGF21 function/expression might induce compensatory changes in FGF19 levels to preserve metabolic homeostasis.[37] Interestingly, rs739320 has previously been related to a more favorable kidney function profile,[38] although the authors did not propose any mechanistic explanation. Now, our findings indicate that rs739320, by decreasing abnormally high FGF19 levels in CKD patients, could lead to better control of glucose homeostasis and regulation of lipid synthesis,[39] which in turn could contribute to the observed beneficial effect on kidney function.

Furthermore, we have shown that genetic variability of the endocrine FGF system may be associated with CV risk in CKD. Most notably, *FGF19* rs1307968 carriers had lower CV

Table 2. Demographic and clinical features of participants that did or did not experience cardiovascular events in the study

	No CVE (n = 767)	CVE (n = 69)	p value
Sex			
Men	467 (60.9%)	52 (75.4%)	0.015
Women	300 (39.1%)	17 (24.6%)	
Age, years	65.3 (14.4)	70.9 (9.8)	0.004
Weight, kg	80.2 (42.6)	79.1 (16.6)	0.234
BMI, kg/m^2	28.8 (5.5)	30.0 (5.1)	0.038
Glucose, mg/dL	119.1 (54.0)	148.5 (61.7)	<0.0001
Total cholesterol, mg/dL	155.6 (38.9)	146.0 (42.7)	0.027
HDL cholesterol, mg/dL	50.4 (32.6)	41.8 (12.7)	0.001
LDL cholesterol, mg/dL	82.7 (62.4)	76.1 (43.8)	0.023
Total calcium, mg/dL	9.4 (3.5)	9.9 (7.6)	0.001
Potassium, mEq/L	4.9 (1.7)	5.0 (0.8)	0.014
Sodium, mEq/L	140.1 (6.6)	140.5 (3.2)	0.896
ACR (mg/g) in urine 24 h	629 (1.108)	876 (1.507)	0.237
Hypertension			
No	154 (20.2%)	9 (13.0%)	0.134
Yes	609 (79.8%)	60 (87.0%)	
History of CVE			
No	572 (74.6%)	39 (59.1%)	0.009
Yes	195 (25.4%)	27 (40.9%)	
Diabetes			
No	454 (59.5%)	18 (26.1%)	<0.0001
Yes	309 (40.5%)	51 (73.9%)	
Smoking			
Non-smoker/former	617 (83.0%)	53 (80.3%)	0.578
Smoker	126 (17.0%)	13 (19.7%)	
Systolic blood pressure, mmHg	141.8 (25.0)	151.3 (34.0)	0.056
Diastolic blood pressure, mmHg	75.1 (14.5)	73.8 (23.3)	0.113
Pulse pressure, mmHg	66.6 (22.6)	77.4 (22.9)	0.002
CKD stage			
Control	176 (22.9%)	5 (7.2%)	<0.0001
CKD 3	77 (10.0%)	15 (21.7%)	
CKD 4–5	304 (39.6%)	17 (24.6%)	
CKD5d	210 (27.4%)	32 (46.4%)	

BMI, body mass index; CVE, cardiovascular event; CKD, chronic kidney disease.
Mean (standard deviation) values or count (percentage) are shown.

event-free survival. This is a novel finding, as there is no information available in the literature regarding this SNP, except for an association with the risk for cleft lip.[40] rs1307968 was included in the study as an intronic tag-SNP, i.e., it represents genetic variability in an area of the gene locus. Therefore, the SNP must likely be in high linkage disequilibrium (LD) with another functional variant on which the observed clinical effect would be dependent. A putative candidate is rs1452459680 C/G, which is in almost complete LD and is located within a transcription factor binding site. We also found a detrimental effect of the *FGF23* rs11063112AA genotype. Consistently, a large study in hemodialysis patients has reported that this variant was associated with CV mortality.[17] We did not observe that this SNP affected FGF23 levels, which increase CV risk; therefore, another underlying mechanism must be considered, e.g., altered mRNA stability, since the SNP lies within the 3′UTR region. A third SNP, *FGFR4* rs31776, was also linked to high CV risk. This variant is located in a splicing region and as such could translate into altered function of the receptor, which could contribute to the observed impact. Our results indicate that this could be a variant worth of further examination, as there are no previous studies on its clinical implications. The last significant genotype was *FGFR4* rs351855 G/A, which produces a Gly388Arg substitution that SIFT and PolyPhen repositories regard as deleterious or possibly damaging. It has been reported that the A-allele is linked to decreased risk of coronary artery disease[41] and stroke,[42] which is in line with our observation that GA carriers were at lower risk of CV events in our cohort. Conversely, Selllier et al.[43] did not find an association of this SNP with CV events in a CKD population, although the authors only considered left ventricular hypertrophy and atherosclerosis. In any case, it was only the heterozygous genotype that was significantly associated with CV risk in our patients, and hence caution must be exerted when extrapolating these results. In any case, these genetic results from a homogeneous, sizable cohort add to the existing evidence, arguing for the utility of early genotyping in the CKD setting.[44]

Finally, the addition of the combined scores representing FGF levels through a two-group cluster, along with the four relevant genetic variants to a CV risk model containing classic risk factors, significantly improved its predicting ability from 71% to 78%. We have in the past shown that both genetics and biomarker concentrations can be used to improve risk models for CKD patients.[45,46] In this case, it should be remarked that although both the SNPs and the combined levels had a profound effect on CV event-free survival, the ROC analysis is based on binary logistic regression (event yes/no), and therefore the time variable is not considered. This may have limited the observed model improvement of 6.6 percentual points, which, while it may not justify by itself its use in clinical routine, provides a strong platform for future biomarker-driven strategies to improve risk stratification and management in CKD patients. In any case, our findings build upon previous evidence[47] that there is much room for improvement in the use of traditional markers such as eGFR and albuminuria in the CKD setting. New biomarkers are urgently needed, as CKD is predicted to become one of the top five causes of death worldwide by 2040, mainly because of its CV complications.[48]

To our knowledge, this is the first study that generates a combined score for all three endocrine FGFs to establish associations with CKD severity and CV risk. Moreover, the simultaneous genetic study allowed us to identify relevant variants for the incidence of CVE, which improved CV risk models already containing the FGFs clusters and other classic risk factors. In summary, we present herein a combined score that integrates FGF19, 21, and 23 circulating concentrations and that could function as a dual-risk biomarker in renal patients, capturing both renal and cardiovascular vulnerability. Furthermore, the combination of

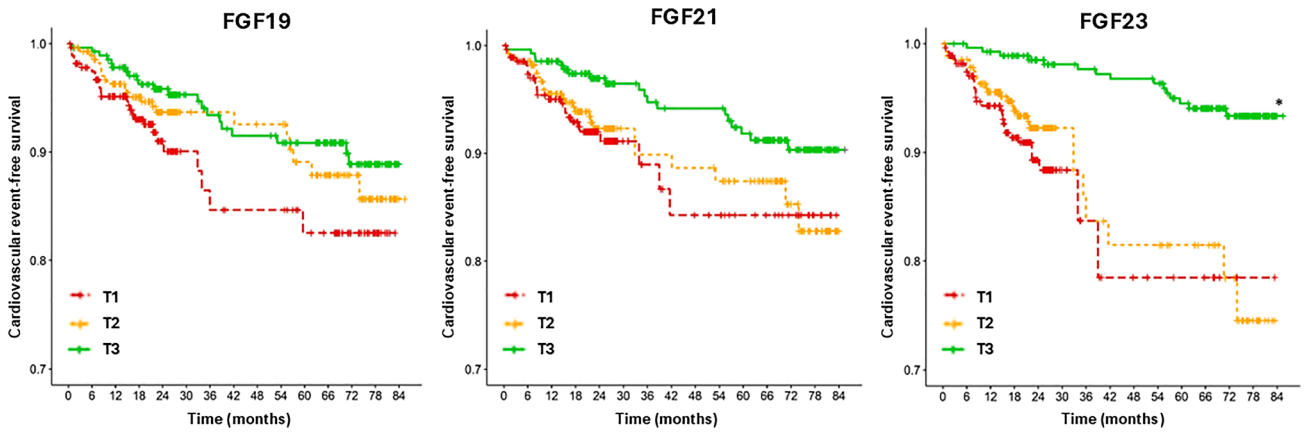

Figure 3. Cardiovascular event-free survival analysis stratified by tertiles of FGFs concentrations

p value for Cox-adjusted models is shown: **p* = 0.029 vs. T1.

this score with the identified CV-related genetic variants, as well as traditional risk factors, holds the potential to significantly improve the management of CV-related complications in CKD.

Limitations of the study

The incidence of CVE was not very high, which could have affected the statistical stability of our analyses. For instance, it forced us to choose a different clusterization method to study event-free CV survival. Also in this regard, only patients with CKD stage ≥3 (with higher likelihood of CVE) were included in the study; while the inclusion of CKD1/2 patients would have been valuable, their participation would have also lowered even further the percentage of CVE registered. Second, while the ELISA methodology was sound, potential batch effects or intra-assay variability cannot be ruled out. Third, genetic material and CV follow-up data were not available for all 1,182 participants, although the resulting cohorts were still sizable.

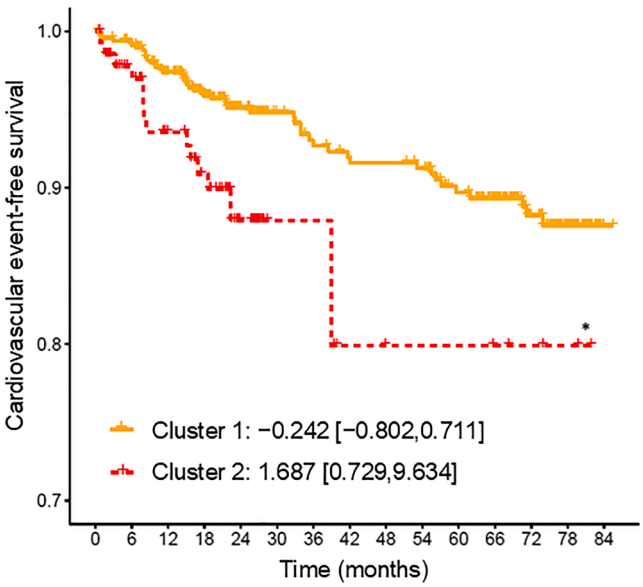

Figure 4. Cardiovascular event-free survival in the two groups yielded by the cluster analysis based on the scores summarizing all three FGF concentrations

p value for Cox-adjusted model: **p* = 0.044.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Guillermo Gervasini (ggervasi@unex.es).

Materials availability

This study did not generate any new material.

Data and code availability

- Source data for this study have been uploaded to Figshare and are openly available at https://figshare.com/articles/dataset/FGFs_in_CKD/29108150?file=55074824 with https://doi.org/10.6084/m9.figshare.29108150.

Table 3. Hazard ratios for genetic variants with significant associations with cardiovascular event-free survival in the population of study

SNP	Genotype	No CVE	CVE	HR (95% CI)	*p* value
FGF19 rs1307968	A/A	76.3%	65.0%	Reference	
	A/G	22.4%	30.0%	1.84 (1.01,3.36)	0.048
	G/G	1.2%	5.0%	5.14 (1.53,17.27)	0.008
FGF23 rs11063112	T/T	55.7%	50.8%	reference	
	T/A	39.0%	39.0%	0.96 (0.52,1.78)	0.894
	A/A	5.4%	10.2%	3.78 (1.48,9.65)	0.006
FGFR4 rs31776	G/G	45.8%	42.2%	reference	
	G/A	45.4%	37.5%	0.93 (0.51,1.72)	0.828
	A/A	8.8%	20.3%	3.02 (1.46,6.25)	0.003
FGFR4 rs351855	G/G	47.2%	63.1%	reference	
	G/A	43.3%	32.3%	0.51 (0.29,0.92)	0.024
	A/A	9.6%	4.6%	0.31 (0.07,1.31)	0.111

CVE, cardiovascular event; HR (95% CI), hazard ratio with 95% confidence intervals.

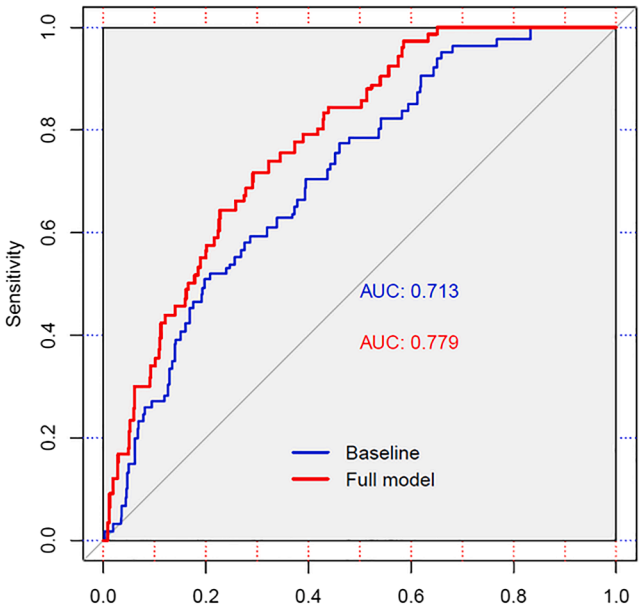

Figure 5. Receiver operating characteristic curves for cardiovascular risk in the population of study

The blue line corresponds to a model containing classic risk factors only, while the red line corresponds to the same model after adding both the relevant genetic variants and the two-group cluster based on the combined score for cardiovascular event-free survival. $p < 0.0001$ for the difference between curves.

- All the different codes for the R packages used are included in the key resources table.
- All other items are also available in the key resources table.

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AUTHOR CONTRIBUTIONS

L.G.-R., data curation and writing of original draft; M.M.-A., formal analysis; S.M.-Z., data curation; C.C., methodology; B.C., A.A., Z.V., and A.F.-A., investigation; F.B., supervision; N.R.R., conceptualization and investigation; G.G., conceptualization, funding acquisition, supervision, and writing—review and editing.

DECLARATION OF INTERESTS

All authors have read the journal's policy on disclosure of potential conflicts of interest. The authors declare no conflict of interest.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Study approval and ethical statement
  - Patient enrollment
  - Main clinical variables
- METHOD DETAILS
  - Determination of FGF circulating levels
  - Genetic analyses
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Human database	This study	https://doi.org/10.6084/m9.figshare.29108150
Software and algorithms		
Simple Plex Runner software (v.3.9.0.28)	Bio-techne	https://www.bio-techne.com/resources/instrument-software-download-center
Haploview software	Broad Institute	https://www.broadinstitute.org/haploview
DMwR2 package	R	https://cran-archive.r-project.org/web/checks/2025/2025-07-10_check_results_DMwR2.html
Nortest package	R	https://cran.r-project.org/web/packages/nortest/
Psych package	R	https://cran.r-project.org/web/packages/psych/refman/psych.html
EFAtools package	R	https://cran.r-project.org/web/packages/EFAtools/index.html
Ckmeans.1d.dp package	R	https://pubmed.ncbi.nlm.nih.gov/27942416/
Cluster package	R	https://cran.r-project.org/web/packages/cluster/index.html
SPSS statistics	IBM	https://www.ibm.com/es-es/products/spss
GPower 3.1	University of Kiel, Germany	https://scispace.com/pdf/gpower-a-general-power-analysis-program-3urzlpnewc.pdf

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study approval and ethical statement

All patients were over 18 years of age and gave written consent for their participation in the study, which was approved by the Ethics Committee of the Badajoz University Hospital (No 220421) and was carried out in accordance with the Declaration of Helsinki and its subsequent revisions.

Patient enrollment

This was a multicentre, observational cohort study, with a baseline cross-sectional biomarker/genetic assessment and prospective follow-up for incident CVE. A total of 1182 Spanish Caucasian subjects were recruited in a 6-year period (2017-2022). Of them, 815 patients were diagnosed with different CKD stages [239 (29.3%) with stage 3; 333 (40.9%) with stage 4/5 and 243 (29.8%) with stage 5d –dialysis], who were enrolled at the Nephrology Service of Badajoz University Hospital and several dialysis units in the province of Badajoz (Badajoz, Zafra and Llerena Hospitals and FRESENIUS clinic). In addition, 367 volunteers with normal renal function were recruited from the Badajoz University Hospital and several primary healthcare centers in Soria (Spain). The percentage of male subjects was 64.4% on patients and 51.9% on controls. Other characteristics can be found in Table 1 of the results.

Inclusion criteria for the patient group were being over 18 years of age and meeting clinical criteria for CKD, with or without a confirmatory biopsy, with an estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m^2. Control subjects must also be over 18 years of age, a eGFR >60 ml/min/1.73 m^2. Transplantation, pregnancy or breastfeeding, active infection, cancer, or acute kidney injury were all considered exclusion criteria.

Main clinical variables

Diagnostic and prognostic stratification of patients was made using the KDIGO classification, the KDIGO table of risk of progression and the CONSORTIUM-CKD equation (www.kidney risk failure.org). Kidney function was assessed by the glomerular filtration rate (GFR) estimated by the CKD-EPI formula. Proteinuria was defined as a value greater than> 500mg (or albuminuria> 300mg) in 24h urine. A biopsy was conducted to confirm diagnosis when proteinuria was over 1g. Clinical records of the study participants were reviewed to retrieve data on renal function, general biochemistry, and CVE experienced during the follow-up, which was possible in 836 out of the 1182 individuals. These were followed until the earliest of CVE, death, or end of study (September 2024). CVE included death from CV cause, acute myocardial infarction, acute coronary syndrome, coronary catheterization requiring angioplasty, coronary bypass, typical angina with positive stress tests, sudden death, stroke, peripheral artery disease and lower limb ischemia.

METHOD DETAILS

Determination of FGF circulating levels

Blood samples were collected and immediately subjected to plasma separation on the day of the participant's visit to the corresponding hospital Service. The determination of endocrine FGFs (FGF19, FGF21 and FGF23) was carried out in an ELLA™ equipment (Bio-Techne, Minneapolis, USA), which is an automated, low-volume, microfluidic ELISA platform with 32-well cartridges that allowed the simultaneous analysis of all three analytes. In brief, 50 μL of diluted plasma with equal volume of sample diluent were added to each well, followed by the addition of 1 mL of wash buffer to the corresponding buffer inlets. Automated immunoassay analyses were initiated via Simple Plex Runner software (v.3.9.0.28), and consisted of system start-up, microfluidic sample splitting, incubation within glass nano-reactor (GNR) channels containing immobilized capture antibody, biotinylated detection antibody and streptavidin dye conjugate, laser excitation of fluorophores and detection of fluorescence signals. Relative fluorescence units for each GNR were converted to FGFs concentrations by inverse fitting to a master calibration curve established by the manufacturer. Because each microfluidic channel has three GNRs, triplicate measurements were produced for each well/sample (average values are given).

Genetic analyses

DNA was purified from whole blood samples using a standard phenol-chloroform extraction method followed by ethanol precipitation. Five genes, namely *FGF19*, *FGF21*, *FGF23*, *FGFR1*, *FGFR4*, were studied using two different approaches. First, tag-SNPs, which define the variability of a specific area in a gene locus, were previously identified. For this, we obtained all the SNPs registered for Europeans for each gene (www.internationalgenome.org) and inserted this information into Haploview software (https://www.broadinstitute.org/haploview) using Ensembl's *VCF to PED converter* tool. Assuming a threshold of $r^2 = 0.8$ and a minimum allele frequency (MAF) of 0.05, the Haploview tagger function generated 46 tag-SNPs by pairwise tagging, which captured 100% of the variability registered in the European population for these five genes. Second, we also included in the analyses 15 variants for which there were reports supporting their functional/clinical impact on endocrine FGFs.[16,19,49] Genotyping analyses were then performed using a customized panel on a QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies, Carlsbad, California, USA) via TaqMan® OpenArray technology. Each run incorporated quality controls, consisting of sample trios sourced from the Coriell Institute Biorepository. These analyses were conducted at the Centro Nacional de Genotipado-Instituto de Salud Carlos III (CeGen-ISCIII; Madrid, Spain).

QUANTIFICATION AND STATISTICAL ANALYSIS

Qualitative variables were described as frequencies, and quantitative variables were summarized as medians with interquartile ranges, since none of them met the normality assumption. To study the association between categorical and quantitative variables Kruskal-Wallis tests were used. Chi squared tests were used for the association between categorical variables. Linear regression models adjusted by meaningful covariates were carried out to establish the association of the FGF plasma concentrations with genetic variants in a codominant model of inheritance. The association of FGF levels or genetic variants with CV event-free survival was assessed in Kaplan-Meier curves and Cox regression models that were adjusted for meaningful covariates. Clinical and demographic covariates incorporated into each model for adjustment were chosen according to clinical criteria and/or univariate analyses. Associations were expressed as hazard ratios (HR) with 95% confidence intervals.

An *exploratory factor analysis* (EFA) was carried out to describe the structure of FGFs levels and underlying latent factors. The outliers were identified by LOF (*local outlier factors*) algorithm, excluding the top 5th percentile from EFA, and the correlation matrix was computed by the *Spearman* method. The suitability of data for the analysis was evaluated using *Bartlett's test of sphericity*, ensuring all inter-item correlations were below 0.8. According to *Kaiser rule* and parallel analysis, the information of all three FGFs could be explained with one factor, whose internal consistency was measured by *Cronbach's alpha*. This factor was used, together with the standardized FGFs concentrations, to create a global score representing the circulating levels of endocrine FGFs for each participant by using the *Thurstone regression-based* method, according to the following formula, where FGF are expressed in pg/mL:

$$score = 0.282 * \frac{FGF19 - 253.2}{209.1} + 0.461 * \frac{FGF21 - 929.8}{1416.2} + 0.799 * \frac{FGF23 - 1277.7}{3553.4}$$

Next, we carried out cluster analyses to group the population according to this score. For the whole population, the analysis by the optimal *univariate k-median* method produced five clusters that were analyzed in relation to the severity of CKD. The score intervals were [-0.911-0.420] for cluster 1, [-0.415,0.132] for cluster 2, [0.140, 1.158] for cluster 3, [1.211,3.856] for cluster 4, and [4.101,9.634] for cluster 5. However, in the subset of patients followed up for CV events, and because of the limited number of events registered, such clusterization resulted in cluster 5 (with the highest scores but a low number of patients) having no events. Therefore, for this particular CV analysis, we decided to apply instead *the partitioning around medoids* method of clusterization, which produced only two groups with a more balanced presence of events. The score intervals were [-0.802,0.711] for cluster 1, and [0.729,9.634] for cluster 2.

To assess the potential use of FGFs concentrations and genetic variability as biomarkers of CV risk in the CKD setting, multivariate logistic regression models were performed. Areas under receiving operating characteristic (ROC) curves (AUCs) were calculated for models containing traditional risk factors, before and after the addition of the two CV cluster groups based on the combined score and the statistically significant genetic variants. AUCs were compared using De Long's test. Models with AUC values from 0.70 to 0.79 are generally considered as having good discriminative power, while those with AUC$\geq$0.80 are excellent. The methodology takes into account ''sensitivity'' (True Positive Rate), which is the proportion of patients with their condition correctly identified, and ''specificity'' (True Negative Rate), the proportion of patients without the condition correctly excluded. Given the relatively low incidence of events, the dataset was balanced by over-sampling techniques prior to the model construction.

Considering the 836 individuals with cardiovascular follow-up, a CVE incidence of 8.3% (69 patients with CVE and 767 without), and a two-sided alpha of 0.05, this sample provides 87.4% power to detect a standardized effect size (Cohen's d) of 0.4 between patients with and without CVE, according to calculations based on the Wilcoxon-Mann-Whitney test (GPower v. 3.1.9.6, Kiel University, Germany).

All the analyses were conducted with different packages in the *R* environment (See key resources table) and IBM SPSS v.22.0 (SPSS Inc., Chicago, IL, USA v.22.0). The threshold for statistically significant associations was set at $p < 0.05$. In the genetic association study, Bonferroni correction for the 46 SNPs assayed lowered the significance threshold to 0.001. Still, we cannot rule out that the statistical power might be limited for detecting small genetic effects.