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Fibroblast Growth Factor 21 Predicts and Promotes Vascular Calcification in Haemodialysis Patients

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Original investigation

Keywords: fibroblast growth factor 21, vascular calcification, haemodialysis, endothelial-to-mesenchymal transition

Posted Date: August 20th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-56680/v1

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Version of Record: A version of this preprint was published at Kidney Diseases on January 1st, 2021. See the published version at https://doi.org/10.1159/000512750.

Abstract

Background: Cardiovascular disease (CVD) is the leading cause of death in haemodialysis (HD) patients. Vascular calcification (VC) is dramatically accelerated and is strongly associated with CVD events and mortality in HD patients. VC coexists with osteoporosis in many studies. Fibroblast growth factor 21 (FGF21) as an adipocytokines is a new hypoglycemic strategy and is inversely related to bone mineral density.

Methods: To evaluate the contribution of FGF21 to VC in HD patients, we preliminary sreened 802 HD patients of two large HD centers in China. At last 388 HD patients were entered this cross-sectional study. We detected circulating FGF21 levels and measured the whole thoracic aorta calcification scores (TACS) and calcification scores of the three segments of thoracic aorta (TA), including ascending thoracic aorta (ATACS), aortic arch (AoACS), and descending thoracic aorta (DTACS) of our 388 HD patients. In addition, we pre-incubated human aortic endothelial cells (HAECs) with FGF21 in the presence or absence of parathyroid hormone (PTH) *in vitro*.

Results: The median serum FGF21 level in HD patients was 11-fold higher than that in healthy controls. Ln(FGF21) was positively correlated with Ln(TACS+1), Ln(ATACS+1), Ln(AoACS+1) and Ln(DTACS+1) respectively in HD patients. Serum FGF21 was independently associated with TACS and ATACS, AoACS, and DTACS. FGF21 combined with age, calcium and intact parathyroid hormone demonstrated a high area under the curve (AUC=0.84) with optimal sensitivity (84%) and specificity (71%) for the prediction of VC in HD patients. Our *vitro* results showed that FGF21 enhanced the calcification effect of PTH on HAECs by increasing calcium deposition and endothelial-to-mesenchymal transition (EndMT).

Conclusions: Circulating FGF21 was notably higher and was a potential predictor and promoter of VC in HD patients.

Trial registration Chinese Clinical Trial Registry, identifier: ChiCTR1900028249. Registered 16 December 2019-Retrospectively registered,http://www.medresman.org.cn/uc/project/projectedit.aspx?proj=5981

Introduction

Mortality rates for patients with end-stage renal disease (ESRD) treated with dialysis remain unacceptably high, and the annual mortality rates remain 15–20%.[1] Cardiovascular disease (CVD) is the leading cause of death among dialysis patients.[2] Vascular calcification (VC) is thought to be a powerful independent risk factor for CVD events and mortality in haemodialysis (HD) patients.[3, 4] VC progresses rapidly in HD patients, and the occurrence of VC among HD patients is 70–90%.[5–7]

The human fibroblast growth factor (FGF) family includes 22 members.[8] FGF23 and FGF21 are endocrine factors that structurally belong to the FGF19 subfamily, are released into circulation and exert systemic action.[9] Serum FGF23 levels increase up to 100–1000 folds in HD patients.[10–12] The involvement of elevated FGF23 in the progression of VC is controversial. Clinical studies indicate a

correlation between higher FGF23 levels and increased aortic calcification.[13, 14] However, other studies have shown that FGF23 had no impact on VC[15, 16] or inhibited the progression of VC[17, 18].

FGF21 is also increased progressively with a decline of renal function.[19, 20] Circulating FGF21 is mainly secreted from the liver as a metabolic regulator of glucose metabolism and lipid metabolism in response to fasting.[21] In obese rodents, it causes weight loss[22] and improves insulin sensitivity[23, 24]. In humans, circulating FGF21 is inversely related to bone mineral density,[25] elevated FGF21 levels are associated with reduced bone strength and with bone mass.[26] FGF21 analogs also increased blood markers of bone loss in two of the human studies.[27, 28] Many studies have demonstrated an independent association between osteoporosis and VC.[29, 30] The role of FGF21 in VC has rarely been reported. Therefore, whether circulating FGF21 is associated with VC warrants further investigation.

Endothelial cells (ECs) can undergo a process known as endothelial-to-mesenchymal transition (EndMT), which involves the loss of endothelial features and the acquisition of a fibroblast-like phenotype, eventually leading to cells with osteogenic potential.[31] However, the role of FGF21 in EndMT and calcium deposition in ECs under uremic stress hasn't been reported.

This cross-sectional study aimed to achieve 4 objectives. The first objective was to quantify the correlation between FGF21 and VC. The second objective was to determine whether FGF21 is a novel risk factor for VC independent of traditional risk factors. The third objective was to examine the predictive effect of FGF21 on VC. The fourth objective was to identify the role and explore the underlying mechanism of FGF21 in the process of VC, especially focused on the EndMT process, in cultured ECs.

Materials And Methods

Study design and population

This cross-sectional study included 802 HD patients from two large HD centres, Nanjing Zhongda Hospital (n = 450) and the First People's Hospital of Changzhou (n = 352), China, from January 2018 to December 2018. The exclusion criteria were described in the flow chart (Fig. 1). Finally 388 HD patients with chest multislice computed tomography (MSCT) examination within half a year and consenting to give blood samples were invited to this study. The enrolled patients underwent stable regular HD using bicarbonate dialysate. Most patients received 4 hours of HD treatment every session 3 times each week. In addition, we obtained data from 20 healthy controls from the physical examination centre of Nanjing Zhongda Hospital.

Clinical and biochemical data collection

Dedails of collection of clinical and biochemical data are provided in the Supplementary Methods.

Measurement of serum FGF21 and FGF23 in HD patients

Serum FGF21 levels were assessed by enzyme-linked immunosorbent assay (ELISA) kits for human FGF21 (Neobioscience, China). Serum FGF23 levels were assessed by ELISA kits for human FGF23 (Joyee Biotechnics, China). The levels of serum FGF21 and FGF23 were measured according to the manufacturers' instructions. Details of the measurements are provided in the Supplementary Methods.

Assessment of vascular calcification

The measurements and definitions of thoracic aorta calcification score (TACS), ascending thoracic aorta calcification score (ATACS), aortic arch calcification score (AoACS) and descending thoracic aorta calcification score (DTACS) in this study are provided in the Supplementary Methods.

Definition of light VC and moderate/severe VC

The calcification scores of all segments were measured. First, HD patients were divided into two groups according to the median TACS (0.77 cm^3): the low TACS group (< 0.77 cm^3) and the high TACS group (≧ 0.77 cm^3). The extents of TAC in HD patients were different (Fig. 2). According to the median TACS (0.77 cm^3) and the high-quartile TACS (4.28 cm^3), the first panel was light VC (< 0.77 cm^3), the second panel was moderate VC (0.77 to 4.28 cm^3), and the third panel was severe VC (> 4.28 cm^3). We defined the light VC as the low TACS group and the moderate/severe VC as the high TACS group in our study (Fig. 2). Second, according to the medians of AoACS (0.43 cm^3) and DTACS (0.25 cm^3), HD patients were divided into the low and the high AoACS groups, the low and the high DTACS groups, respectively. Since the median ATACS score was 0, our HD patients were divided into ATAC-positive group (n = 104) and ATAC-negative group (n = 284).

Cell culture and intervention

To answer the question of whether FGF21 could promote VC, we mainly focused on the effect of FGF21 with or without PTH coincubation on calcium deposition and the EndMT process in cultured human aortic endothelial cells (HAECs). We determined the concentration of FGF21 to stimulate HAECs using a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay. Cell culture and intervention protocols are provided in the Supplementary Methods.

Calcium deposition staining for osteoblast differentiation

The experiment of the calcium deposition in this study are described in the Supplementary Methods.

qPCR for EndMT assays

CD31 is an endothelial cell marker, whereas RUNX2 is an osteoblast marker and FSP1 is a mesenchymal cell marker. A detailed description of the qPCR for EndMT assay in our study is provided in the Supplementary Methods.

Statistical analyses

Continuous variables were shown as mean ± standard or median with interquartile range (25th–75th percentile). Categorical variables were expressed as percentages. Univariate analyses were performed to

compare the differences between the two groups. Student's t-test was used to compare normally distributed data, while the Mann-Whitney U-test was used for non-normally distributed data. Categorical data were compared using the chi-squared test. Bivariate correlation analyses were performed to assess the correlation of TACS with serum FGF21 and other clinical parameters. Pearson's correlation analyses were performed for normally distributed data, and Spearman's correlation analyses were performed for non-normally distributed data. Covariance analyses were used to eliminate the influence of age on TACS. Since FGF21, FGF23, intact parathyroid hormone (iPTH), HD vintage, TACS, ATACS, AoACS and DTACS were non-normally distributed variables, these variables were taken as the logarithm for linear regression analyses. Stepwise multivariate linear regression analyses were performed to evaluate variables independently associated with TACS. Receiver operating characteristic (ROC) curves were performed to calculate the area under the curve (AUC) and compare the prognostic value of every independently associated factor or united factor to VC. All analyses were two-tailed, and $P < 0.05$ was considered to be statistically significant. SPSS Software, version 18.0 was used for all statistical analyses.

Results

Comparison of clinical and laboratory characteristics of HD patients with low or high TACS (Table 1)

The age of HD patients was 57 ± 16 years, and 218 of the patients (56.1%) were male. The overall prevalence of thoracic aorta calcification (TAC) in our HD patients was 70.1% (272 of 388 patients). Serum FGF21 levels in the high TACS group were significantly increased compared to those in the low TACS group, and HD patients in the high TACS group were older, had lower diastolic BP, had lower uric acid, had higher bicarbonate, had higher iPTH, had higher FGF23, had longer dialysis vintage, had a higher incidence of hypertension and a higher incidence of CVD than HD patients in the low TACS group (all $P < 0.05$). There were no additional parameters with significant differences between the two groups (all $P > 0.05$).

Table 1
Characteristics of subjects and comparison of clinical parameters and laboratory data of HD patients between the low TACS group and the high TACS group by univariate analyses.

	All HD patients	Low TACS group	High TACS group	P value
Number(n)	388	194	194	
General data				
Age (years)	57 ± 16	49 ± 14	65 ± 13	**0.000**
Gender (male, %)	56.1	57.80	54.5	0.891
Dialysis vintage (years)	2(0.58,5.65)	1(0.5,4.05)	3(1,7.3)	**0.000**
Body surface area (m2)	1.73 ± 0.23	1.72 ± 0.22	1.74 ± 0.24	0.436
Systolic BP (mmHg)	145 ± 24	144 ± 23	147 ± 25	0.386
Diastolic BP (mmHg)	82 ± 15	84 ± 15	80 ± 14	**0.015**
Blood data				
Hemoglobin (g/L)	98 ± 20	95 ± 22	101 ± 19	**0.007**
Albumin (g/L)	36 ± 6	35 ± 7	36 ± 6	0.362
Uric acid (mmol/L)	379 ± 129	398 ± 135	360 ± 121	**0.016**
Total cholesterol (mmol/L)	4.13 ± 1.15	4.13 ± 1.11	4.12 ± 1.18	0.868
Triglycerides (mmol/L)	1.82 ± 1.36	1.93 ± 1.45	1.69 ± 1.24	0.137
Bicarbonate (mmol/L)	22.75 ± 3.81	22.24 ± 3.76	23.24 ± 3.8	**0.016**
Calcium (mmol/L)	2.26 ± 0.24	2.21 ± 0.22	2.3 ± 0.24	**0.000**
Phosphate (mmol/L)	1.72 ± 0.58	1.75 ± 0.56	1.7 ± 0.61	0.668
Parathyroid hormone (pg/ml)	269 (130,504)	238 (117,420)	297 (145,570)	**0.027**
FGF21 (pg/ml)	217 (96,517)	163 (68,420)	295 (134,682)	**0.000**
FGF23 (pg/ml)	5160 (710,13581)	3030 (493,9230)	7201 (1711,18816)	**0.000**
CT data				
TACS (cm^3)	0.77(0,4.28)	0(0,0.3)	4.28(1.82,8.96)	**0.000**

HD, haemodialysis; FGF21, fibroblast growth factor 21; FGF23, fibroblast growth factor 23; TACS, thoracic aorta calcification score; ATACS, ascending thoracic aorta calcification score; AoACS, aortic arch calcification score; DTACS, descending thoracic aorta calcification score; CVD, cardiovascular disease.

	All HD patients	Low TACS group	High TACS group	P value
ATACS (cm^3)	0(0,0.02)	0(0,0)	0.01(0,0.39)	**0.000**
AoACS (cm^3)	0.43(0,2.11)	0(0,0.16)	2.11(0.83,4.65)	**0.000**
DTACS (cm^3)	0.25(0,1.93)	0(0,0.02)	1.93(0.59,3.83)	**0.000**
Comorbidity				
Diabetes (%)	40.80	37.40	43.70	0.407
Hypertension (%)	86.40	81.00	91.70	**0.002**
CVD (%)	30.30	21.20	37.80	**0.012**
Medicine usage				
Vitamin D (%)	41.70	40.50	38.70	0.336
Calcium supplements (%)	25.20	32.30	19.30	**0.030**
Cinacalcet (%)	15.60	17.20	14.30	0.579

HD, haemodialysis; FGF21, fibroblast growth factor 21; FGF23, fibroblast growth factor 23; TACS, thoracic aorta calcification score; ATACS, ascending thoracic aorta calcification score; AoACS, aortic arch calcification score; DTACS, descending thoracic aorta calcification score; CVD, cardiovascular disease.

Correlation of VC with serum FGF21 levels and other variables in HD patients

Serum FGF21 levels and TACS, iPTH were non-normally distributed data and were logarithmic transformed into Ln(FGF21) and Ln(TACS + 1), Ln(iPTH). Similarly, the ascending thoracic aorta calcification score (ATACS), aortic arch calcification score (AoACS) and descending thoracic aorta calcification score (DTACS) were converted into Ln(ATACS + 1), Ln(AoACS + 1) and Ln(DTACS + 1), respectively. Ln(FGF21) was significantly positively associated with Ln(TACS + 1), Ln(ATACS + 1), Ln(AoACS + 1) and Ln(DTACS + 1) (all $P < 0.001$, Fig. 3). In addition, TACS, ATACS, AoACS and DTACS were all correlated with age, dialysis vintage, diastolic BP, uric acid, calcium, iPTH and FGF23 (all $P < 0.05$, Table 2).

Table 2
Bivariate correlation analyses for the correlations of calcification scores of whole and three segments of thoracic aortic (including TACS, ATACS, AoACS, DTACS) with other variables in HD patients.

Variable	TACS		ATACS		AoACS		DTACS	
	r	P value	r	P value	r	P value	r	P value
Age	0.630	**0.000**	0.373	**0.000**	0.634	**0.000**	0.575	**0.000**
Dialysis vintage	0.308	**0.000**	0.210	**0.000**	0.296	**0.000**	0.296	**0.000**
BSA	0.063	0.237	0.056	0.288	0.086	0.103	0.036	0.503
SBP	0.082	0.108	0.032	0.531	0.092	0.071	0.079	0.123
DBP	-0.212	**0.000**	-0.129	**0.011**	-0.228	**0.000**	-0.179	**0.000**
Hemoglobin	0.151	**0.003**	0.153	**0.003**	0.145	**0.005**	0.116	**0.024**
Albumin	0.082	0.114	0.130	**0.012**	0.068	0.186	0.008	0.119
Uric acid	-0.154	**0.003**	-0.073	0.157	-0.178	**0.001**	-0.117	**0.024**
Total cholesterol	-0.021	0.681	-0.031	0.552	-0.046	0.371	-0.001	0.998
Triglycerides	-0.074	0.154	-0.028	0.628	-0.090	0.080	-0.050	0.337
Bicarbonate	0.009	0.082	0.049	0.347	0.103	**0.045**	0.195	**0.023**
Calcium	0.179	**0.000**	0.136	**0.008**	0.174	**0.000**	0.165	**0.001**
Phosphate	-0.079	0.126	0.033	0.523	-0.109	**0.034**	-0.035	0.504
PTH	0.142	**0.007**	0.132	**0.012**	0.145	**0.005**	0.133	**0.011**
FGF23	0.269	**0.000**	0.176	**0.001**	0.255	**0.000**	0.258	**0.000**

BSA, body surface area; SBP, systolic blood pressure, DBP, diastolic blood pressure; PTH, parathyroid hormone; FGF23, fibroblast growth factor 23; TACS, thoracic aorta calcification scores; ATACS, ascending thoracic aorta calcification scores; AoACS, aortic arch calcification scores; DTACS, descending thoracic aorta calcification scores.

Comparison of serum FGF21 between the ATAC-positive group and the ATAC-negative group, the high AoACS group and the low AoACS group, and the high DTACS group and the low DTACS group

In our HD patients, serum FGF21 levels were significantly higher in the ATAC-positive group (median 433 vs. 182 pg/ml), the high AoACS group (median 305 vs. 159 pg/ml) and the high DTACS group (median 298 vs. 173 pg/ml) than in the ATAC-negative group, the low AoACS group and the low DTACS group respectively (all $P < 0.001$, Fig. 4A, B, C).

Multivariate linear regression analyses for the establishment of independent associated factors for VC

Variables that were different between the high and the low TACS groups in the univariate analyses (age, dialysis vintage, diastolic BP, haemoglobin, uric acid, bicarbonate, calcium, iPTH, FGF21, FGF23, hypertension history, CVD history, calcium supplement usage) and those that were well known as promoters of VC (total cholesterol, triglycerides, phosphate, diabetes history) were entered into a multivariate linear regression analysis. The results showed that age (β = 0.036), Ln(FGF21) (β = 0.192), calcium (β = 0.916) and Ln(iPTH) (β = 0.193) were factors independently associated with Ln(TACS + 1) in HD patients (all P < 0.001, Table 3).

Table 3
Multivariate linear regression analyses for the establishment of factors independently associated with TACS.

Variable	β (95% CI)	P value
Age	0.036 (0.030–0.042)	**0.000**
Ln (FGF21)	0.192 (0.127–0.256)	**0.000**
Calcium	0.916 (0.525–1.306)	**0.000**
Ln (iPTH)	0.193 (0.106–0.281)	**0.000**

iPTH, intact parathyroid hormone; FGF21, fibroblast growth factor 21; FGF23, fibroblast growth factor 23; TACS, thoracic aorta calcification score; CVD, cardiovascular disease.

Additionally, age, calcium, Ln(FGF21) and Ln(iPTH) were independently associated with Ln(ATACS + 1); age, calcium, Ln(FGF21), Ln(iPTH) and Ln(FGF23) were independently associated with Ln(AoACS + 1); and age, calcium, Ln(FGF21) and Ln(FGF23) were independently associated with Ln(DTACS + 1) (Table 4).

Table 4
Multivariate linear regression models of factors independently associated with ATACS, AoACS and DTACS in HD patients.

Variable	Ln(ATACS + 1)[a]		Ln(AoACS + 1)[b]		Ln(DTACS + 1)[c]	
	β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
Age	0.007 (0.004–0.010)	**0.000**	0.029 (0.025–0.034)	**0.000**	0.023 (0.019–0.027)	**0.000**
Calcium	0.284 (0.095–0.437)	**0.003**	0.677 (0.395–0.959)	**0.000**	0.628 (0.352–0.904)	**0.000**
Ln (FGF21)	0.045 (0.015–0.075)	**0.004**	0.123 (0.076–0.169)	**0.000**	0.143 (0.098–0.189)	**0.000**
Ln (iPTH)	0.055 (0.013–0.097	**0.011**	0.134 (0.076–0.192)	**0.000**	-	-
Ln (FGF23)	-	-	0.058 (0.020–0.096)	**0.003**	0.081 (0.045–0.118)	**0.000**

Model a, b, c: All adjusted for age, dialysis vintage, diastolic BP, haemoglobin, uric acid, bicarbonate, calcium, iPTH, FGF21, FGF23, hypertension history, CVD history, calcium supplements usage, total cholesterol, triglycerides, phosphate, diabetes history. iPTH, intact parathyroid hormone; FGF21, fibroblast growth factor 21; FGF23, fibroblast growth factor 23; ATACS, ascending thoracic aorta calcification scores; AoACS, aortic arch calcification scores; DTACS, descending thoracic aorta calcification scores.

Prediction of VC by FGF21, calcium, iPTH and age

To evaluate the discriminative performance of independently associated factors in the prediction of VC, ROC curves were constructed (Fig. 5). The AUC of FGF21 for the prediction of VC was 0.63 ($P < 0.001$) with a high sensitivity (91%) but poor specificity (32%), which achieved statistical significance in HD patients (Table 5). The AUCs of age, calcium, and iPTH for the prediction of VC were 0.78 ($P < 0.001$), 0.60 ($P = 0.001$) and 0.58 ($P = 0.013$), respectively. However, a combined model of FGF21 and age, calcium, iPTH yielded a significant increment in the AUC (0.84, $P < 0.001$) with optimal sensitivity (84%) and specificity (71%) (Table 5).

Table 5
The area under the curve (AUC) of separated and united independently associated factors of TACS in ROC curve analyses.

Variable	AUC (95% CI)	P value	Sensitivity (%)	Specificity (%)
Age	0.78 (0.74–0.83)	**0.000**	59	87
FGF21	0.63 (0.57–0.69)	**0.000**	91	32
Calcium	0.60 (0.54–0.66)	**0.001**	92	22
iPTH	0.58 (0.51–0.63)	**0.012**	60	54
FGF21 + iPTH	0.64 (0.58–0.69)	**0.000**	60	60
Age + Calcium + iPTH + FGF21	0.84 (0.79–0.87)	**0.000**	84	71

FGF21 aggravated the effect of PTH on calcium deposition and EndMT in HAECs (Fig. 6)

The results revealed that compared to the control, the viability of HAECs showed no significant change while the FGF21 concentrations ranged from 5 to 50 ng/ml, but were reduced significantly when the FGF21 concentrations were higher than 100 ng/ml (reduced to 0.86 at 100 ng/ml, $P < 0.05$; 0.79 at 250 ng/ml, $P < 0.01$; 0.54 at 500 ng/ml, $P < 0.01$, Fig. 6A). One previous study demonstrated that PTH at a concentration of 10^{-8} mol/L could induce HAECs EndMT.[32] Additionally, the CCK-8 assay revealed that PTH (10^{-8} mol/L) alone and PTH (10^{-8} mol/L) + FGF21 (50 ng/ml) had no significant influence on HAECs viability (all $P > 0.05$) (Fig. 6A). Therefore, FGF21 concentrations of 50 ng/ml, PTH concentrations of 10^{-8} mol/L, and PTH (10^{-8} mol/L) + FGF21 (50 ng/ml) were applied in our subsequent experiments.

Alizarin Red staining (ARS) showed that calcium deposition was observed in cultured HAECs stimulated by PTH alone and was even aggravated by PTH + FGF21; however, no calcium deposition was observed in HAECs stimulated by FGF21 alone. (Fig. 6B) This result suggested that FGF21 aggravated the calcification effect of PTH on HAECs.

Next, we evaluated the effect of FGF21 on the EndMT process of HAECs. Compared with the control, reduced expression of CD31 mRNA (Fig. 6C) in parallel with increased expression of RUNX2 mRNA (Fig. 6D) and FSP1 mRNA (Fig. 6E) was observed in HAECs stimulated by PTH. Our results indicated that PTH alone induced EndMT. However, FGF21 alone had no significant effect on the EndMT process. The stimulation of PTH + FGF21 in HAECs aggravated the effect of PTH alone on EndMT. More interestingly, these results were consistent with the ARS results mentioned above.

Discussion

To the best of our knowledge, this study is the first to show that serum FGF21 levels were significantly and independently correlated with VC in HD patients. Furthermore, FGF21 combined with age, calcium

and iPTH showed optimal sensitivity (84%) and specificity (71%) for the prediction of VC in HD patients. Additionally, we first demonstrated that FGF21 aggravated the effect of PTH on calcium deposition and EndMT in HAECs. Taken together, our *vivo* and *vitro* results indicated that FGF21 was a novel predictor and potential promoter of VC in HD patients.

CKD is an international public health epidemic and increases annually.[33] The presence and extent of VC is notably higher in patients with CKD than in the general population, even compared with patients at high risk of CVD but with normal renal function.[34] Thoracic aorta calcification (TAC) has been considered an independent predictor of CVD and mortality.[35] The KDIGO CKD-MBD guidelines have indicated that electron beam computed tomography (EBCT) and multislice computed tomography (MSCT) are the gold standards and the most sensitive methods for the detection and quantification of VC.[36] In this study, we used chest MSCT for TAC evaluation and found that the prevalence of TAC in HD patients was 70.1%, which was similar to other epidemiological findings.[37, 38]

FGF21 is mainly secreted by the liver and adipose tissue in the circulation, functioning as a hormone capable of modulating systemic glucose and lipid metabolism.[39] Many studies have indicated that serum FGF21 levels were negatively correlated with renal function and were elevated 8–15 folds in patients receiving dialysis in comparison to controls.[19, 20] In our study, the median serum FGF21 level in HD patients was elevated 11-fold compared with healthy controls (Table 6). Serum FGF21 levels were positively correlated with TACS in our HD patients, which suggested serum FGF21 increased with the extent of TAC in HD patients. Multivariate regression analyses showed that only age, FGF21, calcium and iPTH were independent factors associated with TACS. Even after adjusting for the covariate of age, the independent association between serum FGF21 and TACS remained. All the above-mentioned results suggested a key role of FGF21 in TACS in HD patients.

Table 6
Comparison of clinical parameters and laboratory data between HD patients and healthy controls.

	HD patients	Healthy controls	P value
Number (n)	388	20	-
Age (years)	57 ± 16	53 ± 17	0.136
Gender (males)	56.10%	60.00%	0.248
Dialysis vintage (years)	2 (0.58, 5.65)	-	-
Body mass inde x(kg/cm^2)	1.73 ± 0.23	1.72 ± 0.61	0.873
Systolic BP (mmHg)	145 ± 24	134 ± 16	**0.023**
Diastolic BP (mmHg)	82 ± 15	76 ± 9	**0.016**
Hemoglobin (g/L)	98 ± 20	145.55 ± 16.06	**0.000**
Albumin (g/L)	36 ± 6	45.93 ± 4.56	**0.000**
Uric acid (mmol/L)	379 ± 129	302.45 ± 79.40	**0.000**
Cholesterol (mmol/L)	4.13 ± 1.15	4.69 ± 0.85	**0.008**
Triglycerides (mmol/L)	1.82 ± 1.36	1.25 ± 0.72	**0.026**
Dicarbonate (mmol/L)	22.75 ± 3.81	24.90 ± 1.54	**0.000**
Calcium (mmol/L)	2.26 ± 0.24	2.34 ± 0.15	**0.048**
Phosphate (mmol/L)	1.72 ± 0.58	1.14 ± 0.25	**0.000**
Intact parathormone (pg/ml)	269 (130, 504)	-	-
FGF21 (pg/ml)	217 (96, 517)	20 (2, 287)	**0.000**
FGF23 (pg/ml)	5160 (710, 13581)	293 (93, 529)	**0.000**

HD, haemodialysis; FGF21, fibroblast growth factor 21; FGF23, fibroblast growth factor 23.

Because computed tomography (CT) examination is a radiologicaly hazardous to humans and calcification assessment software is not used universally, we recommend serum biomarkers for the prediction of VC in HD patients. Our clinical study revealed that serum FGF21 was obviously increased in HD patients and was positively related to the extent of TAC. Moreover, FGF21 combined with age, calcium and iPTH formed a combined predictor whose AUC for the prediction of VC in HD patients was 0.84 ($P < 0.001$) with high sensitivity and specificity. Taken together, these results indicate that the detection of serum FGF21 with age, calcium and iPTH could be used as a preliminary screening method for the prediction of VC in HD patients. This discovery can provide an easier way to identify HD patients with

moderate/severe VC and can avoid extra radiation and cost. It may also provide a new therapeutic target for the treatment of VC in HD patients.

A study compared HD patients and sex-age matched subjects without CKD revealed that HD patients were more likely to exhibit a greater arc of calcification at the culprit of VC—a higher frequency of intimal thin calcium and calcified nodules.[40] Secondary hyperparathyroidism (SHPT) is a common complication of CKD patients.[41] In a study of nearly 1300 US dialysis centres and 39,000 HD patients, more than 11% of patients had an iPTH > 600 pg/ml.[42] PTH could induce the transition of ECs to chondrogenic cells via EndMT.[43] In our *vitro* study, FGF21 alone could not induce EndMT and calcium deposition in HAECs, but when FGF21 coincubated with PTH, these two processes were aggravated compared with PTH incubated alone, suggesting that FGF21 was a potential promoter of intimal calcification in an environment where SHPT coexisted. Cao Fang reported that FGF21 attenuates the calcification of vascular smooth muscle cells (VSMCs) *in vitro*,[44] but our study revealed that FGF21 can aggravate the calcification capacity of PTH on HAECs, which suggested that the pro-calcification effects of FGF21 on ECs could overcome its anti-calcification effects on VSMCs. This is further confirmed by our clinical data that FGF21 combined with iPTH is better for the prediction of VC than iPTH alone (Supplementary Table S2). Hence, this study provided both *in vivo and in vitro* evidence that FGF21 was one of the key factors that predicted VC and promoted intimal calcification in HD patients.

The most important significance and contributions of this study are mainly in three aspects. First, we screened 388 of 802 HD patients in two large HD centres in China; comprehensively evaluated the calcification levels of the whole thoracic aorta and different segments of the thoracic aorta; and, for the first time, found an independent correlation of FGF21 with VC. Second, FGF21 combined with age, calcium and iPTH exerted a high AUC with a high sensitivity and specificity for the prediction of VC in HD patients. Third, our results indicated for the first time that FGF21 can amplify the role of PTH in promoting calcium deposition and EndMT *in vitro*, further supporting the results of our clinical data.

Despite the added precautions, there are limitations to the current study. First, the relatively small sample size and the cross-sectional study design preclude the determination of cause and effect. This limitation highlights the need for adequately powered RCTs and observational studies to further confirm the findings presented here. Second, because the clinical results may differ owing to the ethnicity of the participants, our results do not extend to other ethnic groups. Third, our study is a partly retrospective study, all the HD patients in our study had MSCT scans before the study, which may lead to a high risk of selection bias. The signalling pathway and detailed mechanism of FGF21 combined with PTH to promote calcium deposition and EndMT merit further study.

Conclusions

We reported for the first time that serum FGF21 contributes to VC and may serve as a novel predictor for the presence and extent of TAC in HD patients. Furthermore, FGF21 enhanced the calcification effect of

PTH on HAECs. These findings provide novel insight into FGF21 on VC in HD patients and may indicate a new therapeutic target for this life-threatening disorder.

Abbreviations

AoACS: aortic arch calcification scores; ATACS: ascending thoracic aorta calcification scores; DTACS: descending thoracic aorta calcification scores; EndMT: endothelial-to-mesenchymal transition; FGF21: fibroblast growth factor 21; FGF23: fibroblast growth factor 23; HAECs: human aortic endothelial cells; TAC: thoracic aorta calcification; TACS: thoracic aorta calcification scores.

Declarations

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Zhongda Hospital, Affiliated to Southeast University (approval number: 2019ZDKYSB191, 05/08/2019).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request and approval by the principal investigator.

Competing interests

None of the authors has any conflict of interest to declare.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81700618), the Natural Science Foundation of Jiangsu Province (BK20181487), and a Southeast University High-Level Thesis Project to Bin Wang; the Medical research project of Jiangsu Commission of Health (H2019061); the National Key Research Programme (2018YFC130046, 2018YFC1314000) and the Clinic Research Centre of Jiangsu Province (BL2014080); the Key Subject Construction Programme for Nephrology of Suzhou (Szxk201807); Jiangsu Provincial Health and Family Planning Commission (KY2018105). The authors would like to thank all medical staff in our hospital for their help with data collection.

Authors' contributions

Liqiong Jiang and Bin Wang had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study design: Bin Wang and Bicheng Liu. Acquisition, analysis, or interpretation of data: all authors. Drafting of the manuscript: Liqiong Jiang, Min Li and Bin Wang. Critical revision of the manuscript for important intellectual content: all authors. Statistical analysis: Liqiong Jiang, Yucheng Han. Obtained funding: Bin Wang, Liqiong Jiang, Bicheng Liu, Min yang.

Acknowledgements

The authors are grateful to all participants for their times and efforts.

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Figures

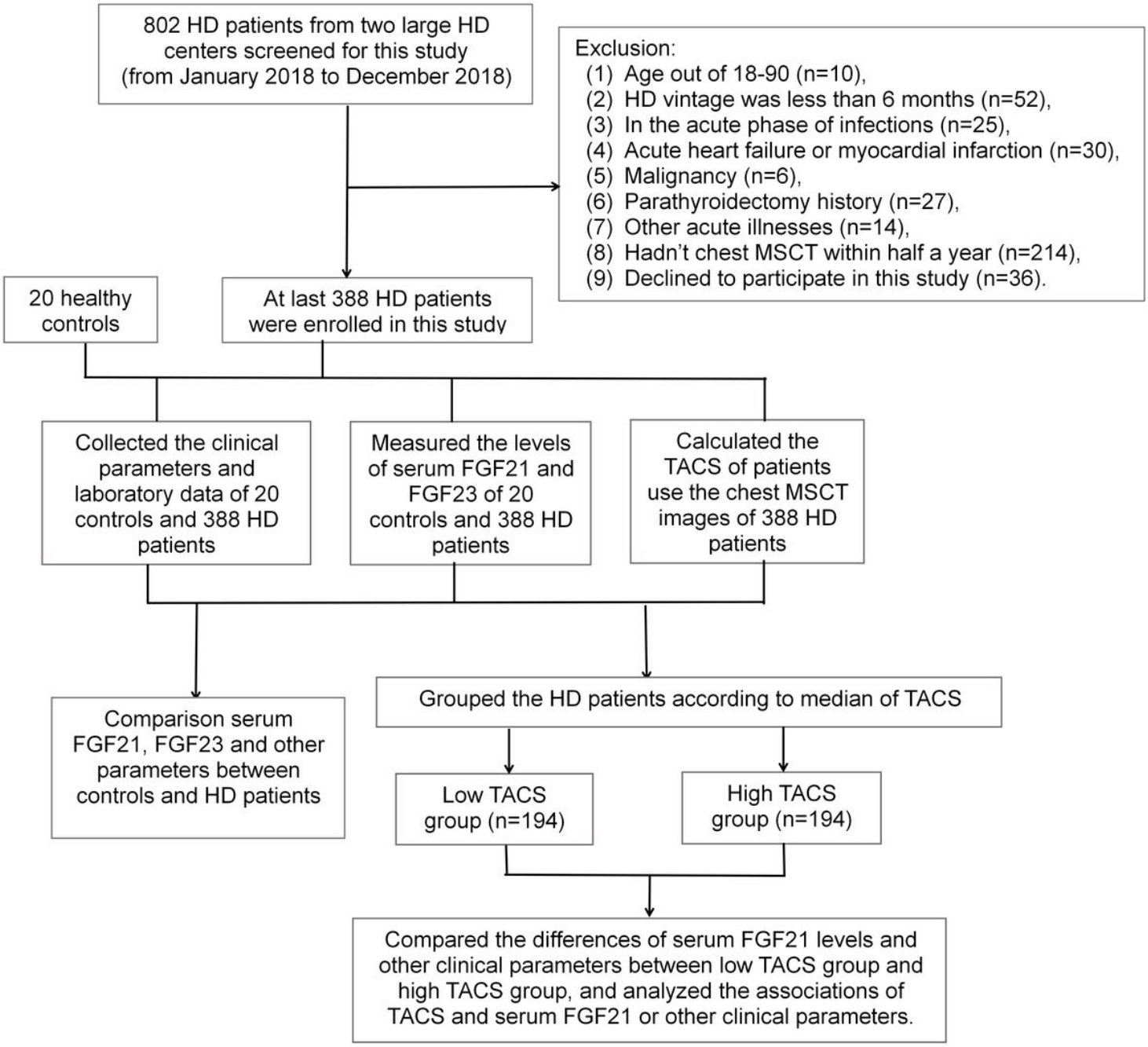

Figure 1

Flow chart of the cross-sectional study.

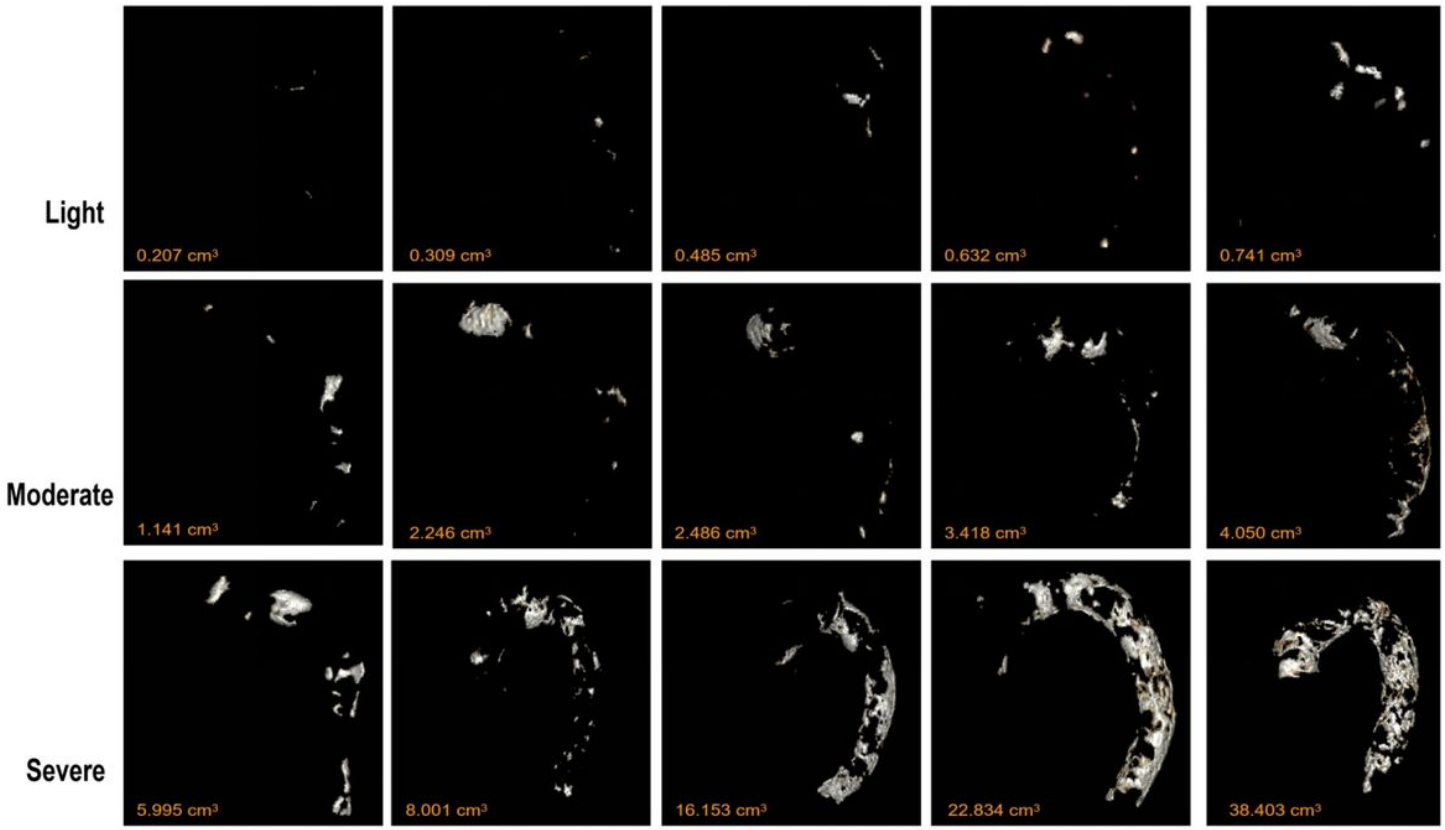

Figure 2

Three-dimensional images of the thoracic aorta calcification (TAC) and different extents of TAC: the first panel the light VC; the second panel the moderate VC; and the third panel severe VC. VC, vascular calcification.

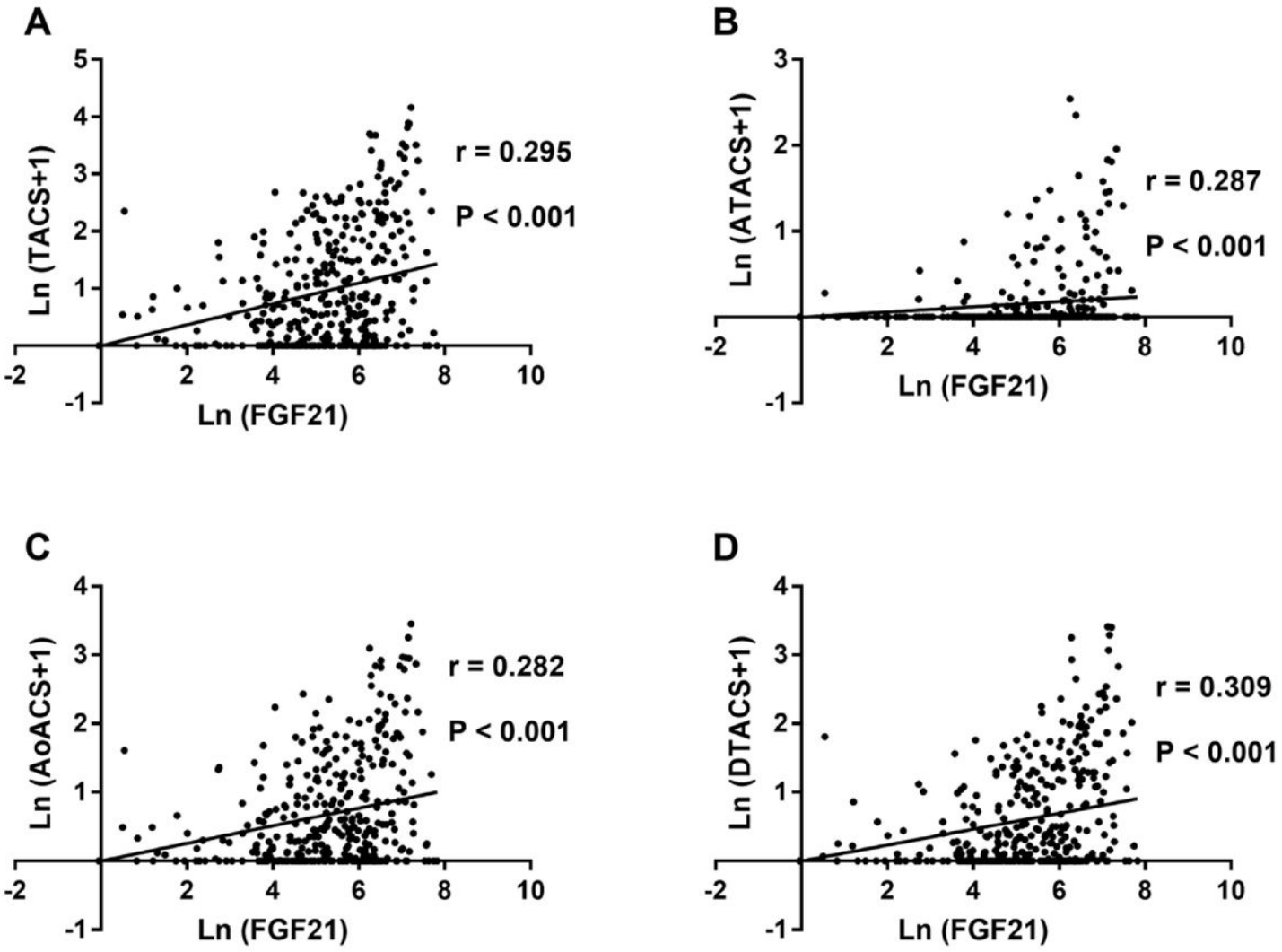

Figure 3

Correlation of serum FGF21 with calcification scores of the whole and three segmented thoracic aortas in HD patients: Ln(FGF21) was significantly positive correlated with: (A) Ln(TACS+1); (B) Ln(ATACS+1); (C) Ln(AoACS+1); (D) Ln(DTACS+1). Ln, logarithmic transformation.

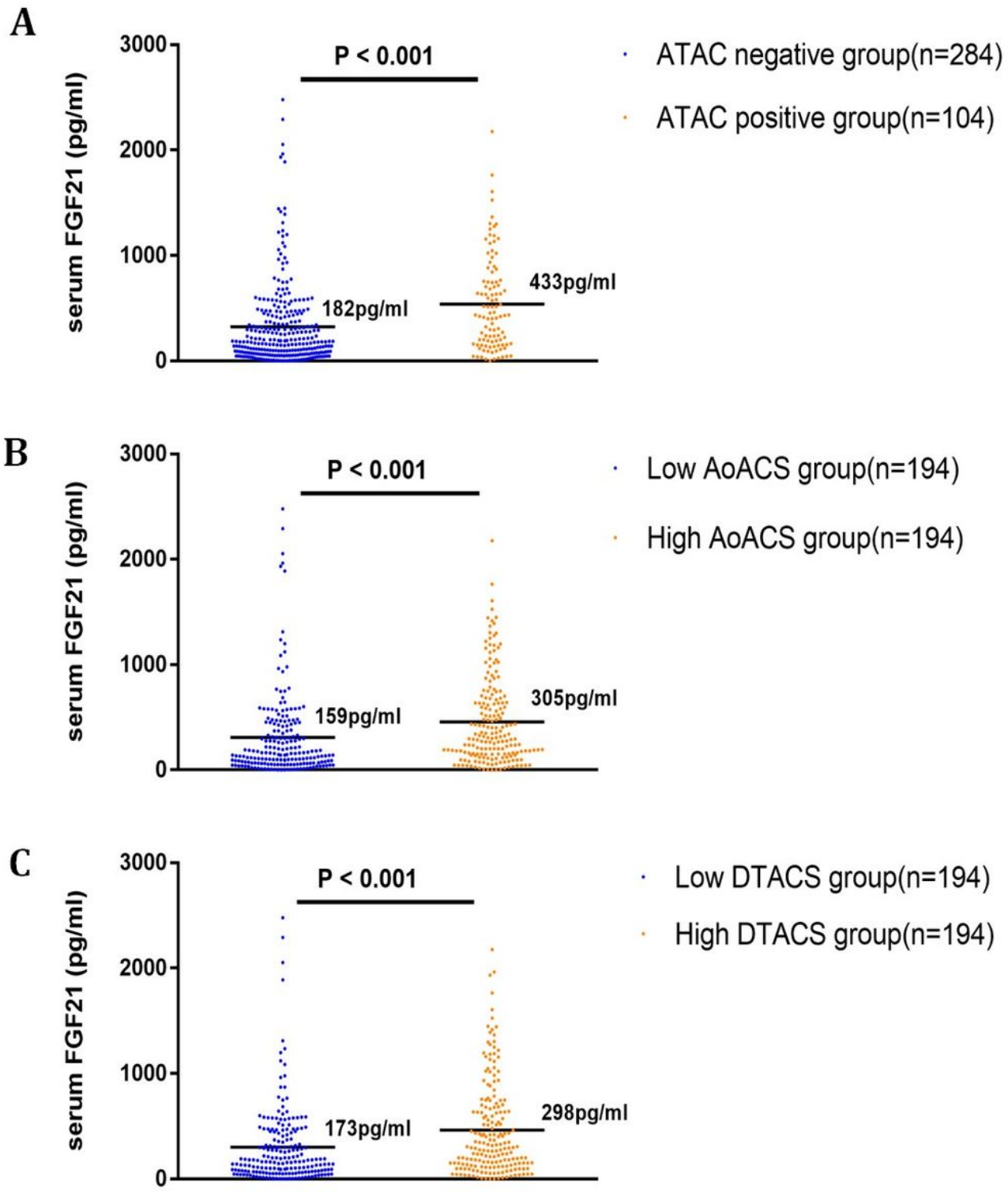

Figure 4

Comparison of serum FGF21 between the ATAC-positive group and the ATAC-negative group, between the high AoACS group and the low AoACS group, and between the high DTACS group and the low DTACS group: Serum FGF21 levels were significantly higher in: (A) the ATAC-positive group than the ATAC-negative groups; (B) the high AoACS group than the low AoACS group; (C) the high DTACS group than the low DTACS group.

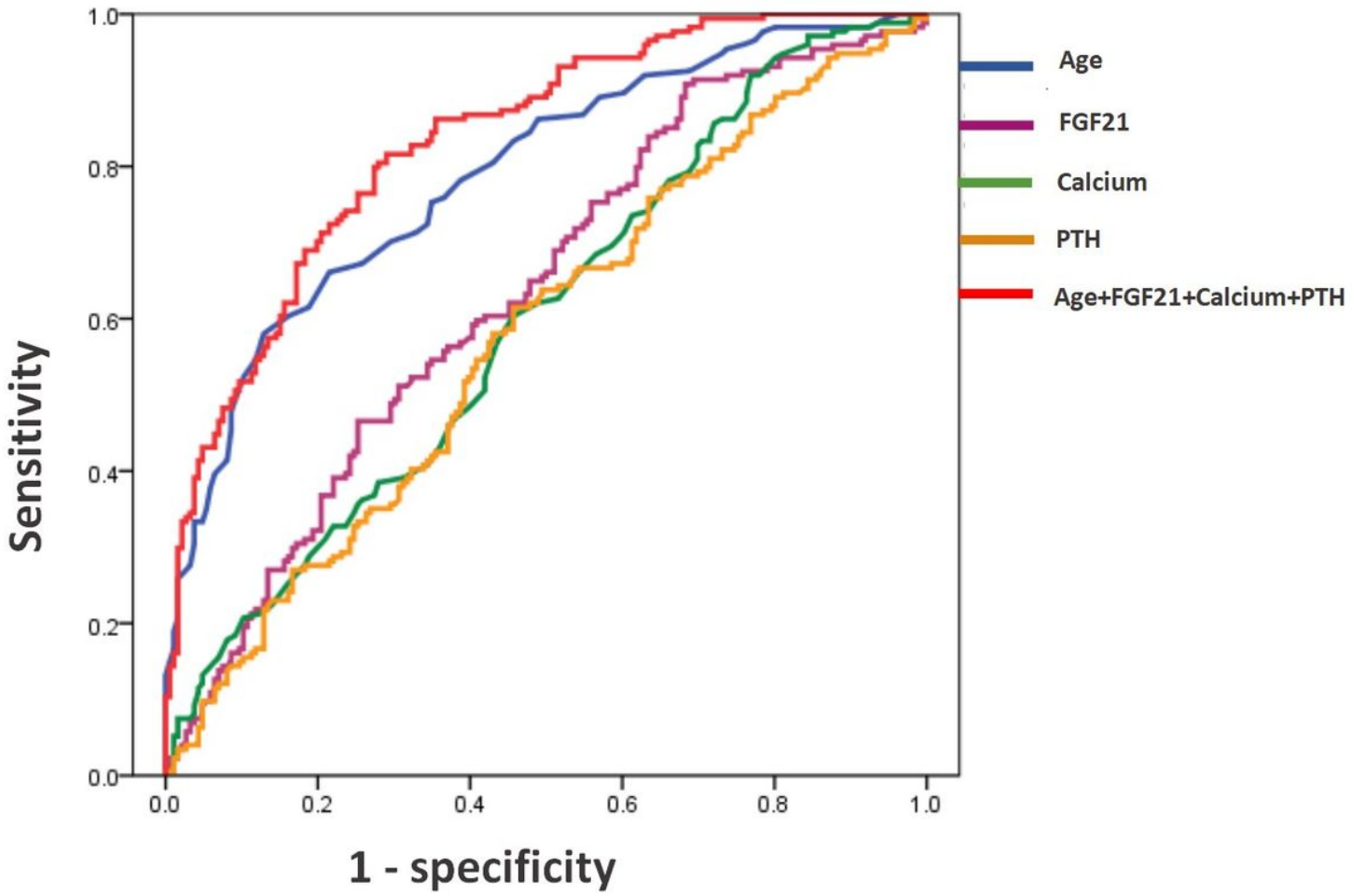

Figure 5

Receiver operating characteristic (ROC) curves analyses of age, iPTH, FGF21, Calcium, and the combined model of age, FGF21, calcium and iPTH for prediction of VC in HD patients: Blue line, age (AUC=0.78, P<0.001); green line, FGF21 (AUC=0.63, P<0.001); purple line, Calcium (AUC=0.60, P=0.001); brown line, iPTH (AUC=0.58, P=0.013) ; orange line, FGF21+iPTH (AUC=0.64, P<0.001); red line, age+FGF21+iPTH+FGF23 (AUC=0.83, P<0.001).

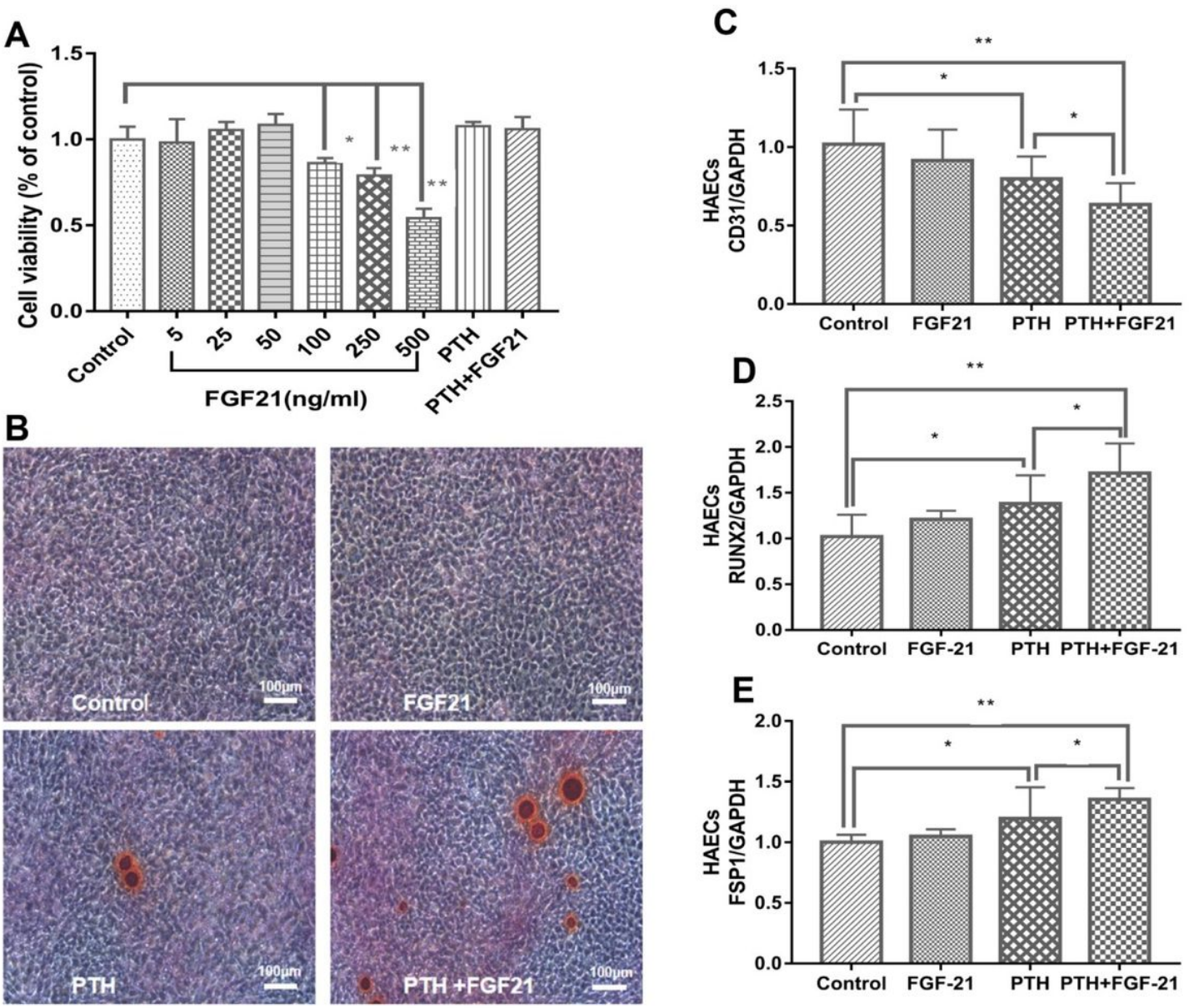

Figure 6

FGF21 enhanced the calcification effect of PTH on HAECs via calcium deposition and EndMT. (A) CCK-8 assay for HAECs viability in different conditions. (B) Alizarin Red staining on HAECs stimulated by FGF21 with or without PTH coincubation. Bright red or orange staining indicates a positive result for calcium deposition. (C) Expression of CD31 mRNA in HAECs stimulated by FGF21 with or without PTH co-incubation. (D) Expression of RUNX2 mRNA in HAECs stimulated by FGF21 with or without PTH co-incubation. (E) Expression of FSP1 mRNA in HAECs stimulated by FGF21 with or without PTH coincubation. *P<0.05, **P<0.01.

Supplementary Files

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