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**Association of Bone-derived Biomarkers with Vascular Calcification in Chronic Hemodialysis Patients**

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**Abstract**

Background: Abdominal aortic calcification (AAC) is commonly observed in chronic dialysis patients and is associated with cardiovascular and all-cause mortality. We investigated the factors associated with AAC and analyze the relationship between bone-derived biomarkers and AAC.

*Methods*: We enrolled 227 stable hemodialysis patients. Vascular calcifications were assessed using lateral lumbar radiography of the abdominal aorta. Demographic data were collected and serum levels of biochemical and bone-derived biomarkers, including sclerostin, Dickkopf-1 (DKK-1), and fibroblast growth factor 23 (FGF23), were measured.

Results: One hundred sixty-one patients (71.0%) had AAC. Patients with AAC score ≧13 were older, with higher body mass indexes (BMI), serum calcium, calcium phosphate product, high-sensitivity C-reactive protein (hsCRP), and FGF23 levels. Sclerostin and DKK-1 levels were inversely associated with AAC severity, and FGF23 was directly related to vascular calcification. Hypertension, vascular disease, hsCRP, FGF23, and sclerostin were independent AAC determinants.

*Conclusions:* Chronic hemodialysis patients have a high prevalence of vascular calcifications. Levels of circulating sclerostin, DKK-1, and FGF23 were related to AAC severity. Sclerostin and FGF23 were independently associated with AAC.

**Key words:** Vascular calcification; DKK-1; Sclerostin; FGF23; Hemodialysis

## 1. Introduction

Vascular calcification is not a new phenomenon. Thompson et al. found its existence in approximately one third of mummies from the preindustrial population that spanned over 4000 years [1]. Furthermore, patients with chronic kidney disease (CKD) have a higher prevalence (even more than 80 percent) and increased severity compared to the general population [2,3]. Excessive vascular calcification among dialysis patients plays a prognostic and contributive role in subsequent cardiovascular events and all-cause mortality [4,5].

In past decades, scientists previously identified the traditional detrimental determinants of vascular calcification among CKD patients, such as increasing age, length on dialysis therapy, net positive calcium and phosphate balance, excessive vitamin D therapy, chronic inflammation, diabetes, and dyslipidemia [6]. More recently, the complex pathophysiological mechanism of vascular calcification, which is regarded as one of the major abnormalities of the CKD-mineral bone disorder, has also been identified as an active extra-endochrondral ossification process [7]. The dysregulation of osteogenic signaling promotes the conversion of vascular smooth muscle cells to osteo/chrondrocytic-like cells, followed by the development of dystrophic mineralization and vascular calcification [8].

Sclerostin and Dickkopf-1 (DKK-1), which are secreted by osteocytes, have both been identified as master antagonists to the Wnt-ß-catenin signaling pathway. By binding to LPR-5/6 receptors, they are capable of inhibiting [9]. There is considerable evidence of the

crucial role of the Wnt-ß-catenin signaling pathway in the regulation of bone homeostasis and vascular biology [10-12]. Among the CKD population, circulating sclerostin levels were elevated while the glomerular filtration rate declined, due to poorly understood mechanisms involving either increased production or renal accumulation [13]. However, the role of sclerostin and DKK1 in vascular calcification remains inconclusive [14-17]. Fibroblast growth factor 23 (FGF23), another bone-derived molecule, is a heparin-binding 30kDA protein that is responsible for enhancing phosphate excretion along with the cofactor klotho, which is mediated by inhibiting expression of renal sodium-phosphate transporters as well as synthesis of calcitriol [8]. Despite the noxious effect of FGF23, which contributes to left ventricular hypertrophy [18], information regarding the relationship between FGF23 and vascular calcification currently involves diverse perspectives [19-20].

Recently, the Kidney Disease Improving Global Outcomes (KDIGO) clinical practice guidelines recommend the lateral abdominal radiograph as a tool for detecting the presence of vascular calcification in CKD patients [21]. Through the utilization of a semi-quantitative method for assessing vascular calcification in the plain lumbar spine film, Kauppila et al. concluded that this was a simple, reproducible, and less expensive approach to detecting the severity and location of aortic atherosclerosis [22].

## 2. Materials and methods

### 2.1. Patient selection

Adult stable uremic patients who have been on maintenance hemodialysis (4 h/session and 3 sessions a week) using bicarbonate-based dialysate with calcium concentrations of 2.5-3.5 mEq/l for at least 3 months were enrolled in the study. Subjects with renal transplantation history or life-threatened comorbid conditions such as malignancy and active infection occurring within the recent 3 months were excluded. Written informed consent was obtained from all participants. The study was reviewed and approved by the Institutional Review Board of Chang Gung Memorial Hospital. Two hundred and twenty seven stable chronic hemodialysis patients were recruited. The underlying causes for uremia included chronic glomerulonephritis (n=75; 33%), diabetes (n =104; 46%), hypertension (n =37; 16%), polycystic kidney disease (n=5; 2%), and unknown etiology (n=6; 3%). Demographic data regarding age, sex, body mass index (BMI), and dialysis duration were reviewed. Comorbidities such as diabetes, hypertension, and vascular disease (defined as previous stroke, coronary artery disease, or peripheral vascular disease) were also recorded.

### 2.2. Laboratory measurements

Fasting blood samples were obtained prior to mid-week dialysis sessions. Before radiographs were obtained, biochemical data collected in the previous three months were recorded and averaged, including corrected serum calcium, phosphorous, albumin, intact

parathyroid hormone (iPTH), alkaline phosphatase, total cholesterol, low-density lipoprotein (LDL), and high-sensitivity C-reactive protein (hsCRP) levels. The iPTH levels were measured using direct chemiluminescent immunoassay (Siemens Healthcare Diagnostics Inc., Erlangen, Germany). Serum levels of sclerostin, DKK-1, and FGF23 were determined in duplicate by using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc. and Immutopics Inc.). The minimum detectable doses of sclerostin, DKK-1, and FGF23 were 1.74 pg/ml, 0.94 pg/ml, and 6 pg/ml, respectively. Their corresponding reportable intra- and interassay CVs were 2.0% and 8.2%, 4.2% and 7.6%, and 2.0% and 3.5%, respectively.

**2.3. Assessment of AAC and severity grading**

Lateral lumbar radiography was used to assess AAC in all study subjects. The presence and severity of AAC were calculated as a validated semi-quantitative scoring system according to the method used by Kauppila et al. [22] and interpreted by two nephrologists who were blinded to the clinical data. Briefly, the Kauppila index (AAC score; range 0-24) represents the sum of the calcification grade of the anterior and posterior abdominal aortic walls, after dividing into four sections, which corresponded to the lumbar spine from the first (L1) to the fourth (L4) segments. Individual aortic wall segments were scored from 0 to 3 (0: no calcification; 1: uneven punctuate calcification; 2: regional linear calcification; 3:

longitudinal calcification spanning more than two-thirds of the vertebra segment). AAC severity was classified as absent (AAC=0), mild (1≦AAC score≦5), moderate (6≦AAC score≦12), or severe (AAC score≧13).

### 2.4. Statistical analysis

We used the PASW statistics software ver 18.0 (SPSS Inc) for all statistical analyses. All categorical data were expressed as absolute numbers (frequency), while all continuous data were presented as mean values ± SD or median (inter-quartile range, IQR), depending on whether the data were normally distributed, as examined by the Kolmorgorov-Smirnov Z test. Spearman correlation analysis, a nonparametric test, was used to assess the relationship among bone-derived biomarkers, clinical and biochemical factors, and AAC scores. All subjects were subsequently stratified into four groups, depending on the AAC scores. In order to compare the different AAC severity groups, the chi-square test was used for categorical variables and either the one-way analysis of variance (ANOVA) or the Kruskal Wallis test was utilized for the continuous variables with normal or skewed distribution, respectively. Meanwhile, the levels of bone-derived biomarkers among the four groups were compared using the post-hoc Bonferroni-corrected Mann-Whitney U test. Additionally, in order to explore the independent factors that are attributed to the AAC scores, variables with a $p < 0.1$ in univariate linear regression analysis (including age, BMI, diabetes, hypertension, vascular

disease, calcium, calcium phosphate products, albumin, hsCRP, sclerostin, DKK-1, FGF23) were selected for multiple linear regression analysis. A two-sided $P< 0.05$ indicated statistical significance.

## 3. Results

### 3.1. Subject characteristics and distribution of abdominal aortic calcification

The mean age of the enrolled patients was 63.0 ± 10.1 y and male patients accounted for 45% of all subjects. The mean duration of hemodialysis therapy was 8.6 ± 5.8 y. Hypertension and vascular disease were noted in 71% and 31% of the study population, respectively. Fig. 1 illustrates the distribution of AAC in our patients, according to the affected individual and total involved segments. Sixty-six subjects (28.1%) did not show evidence of AAC. The L4 was the most affected individual segment (65.6%), followed by L3 (62.6%), L2 (43.2%), and L1 (34.8%). The severity of AAC varied between the four segments, with L4 having the highest AAC scores (mean AAC score for each segment: L4=2.5; L3=2.3; L2=1.2, and L1=0.9, $P< 0.0001$). In addition, all four segments were involved in 28.9% subjects, while 3, 2, and 1 segment, were involved in 14.9%, 17.9%, and 9.8% subjects, respectively.

### 3.2. Comparison of AAC severity among groups

Results of the comparison of the different AAC severity among patients are summarized in Table 1. Patients without calcification were younger and had lower BMI, while groups with severe calcification had higher proportions of hypertension and vascular disease. Their blood levels of calcium and calcium phosphate products were significantly higher than those of the other groups. Furthermore, these patients had the highest hsCRP levels. Patients with severe AAC also demonstrated lower sclerostin and higher FGF23 levels. After adjusting for the other factors, there were significant differences in sclerostin and FGF23 levels between the different groups (Fig. 2). Interestingly, we found a decreasing trend in the albumin and DKK-1 levels with increasing AAC severity. The sex distribution, dialysis duration, prevalence of diabetes, levels of phosphorous, iPTH, alkaline phosphatase, and lipid profile were similar between the four groups.

### 3.3. Relationship between bone-derived biomarkers and AAC

As shown in Fig. 3, serum sclerostin levels were directly related to DKK-1 levels and had an inverse association with FGF23. No significant relationship was observed between DKK-1 and FGF23 levels. With regard to other bone mineral metabolism parameters, sclerostin was negatively associated with calcium ($r = -0.139$; $P = 0.04$), phosphorous ($r = -0.153$; $P = 0.023$), calcium phosphate products ($r = -0.202$; $P = 0.003$), iPTH ($r = -0.491$; $P < 0.0001$), and alkaline phosphatase ($r = -0.341$; $P < 0.0001$). The FGF23 levels had positive associations

with calcium ($r = 0.517$, $P < 0.0001$), phosphorous ($r = 0.473$, $P < 0.0001$), calcium phosphate product ($r = 0.599$, $P < 0.0001$), iPTH ($r = 0.506$, $P < 0.0001$), and alkaline phosphatase ($r = 0.121$, $P = 0.025$). No significant association was found between DKK-1 and biomarkers of bone mineral metabolism. There was no significant relationship between hsCRP and bone-derived biomarkers. Levels of sclerostin, DKK-1, and FGF23 correlated significantly to AAC scores (Fig. 4).

### 3.4. Linear regression analysis of determinants of ACC

Table 2 depicts the results of multivariate linear regression analysis of the AAC scores. Age; hypertension; vascular disease; and levels of hsCRP, sclerostin, and FGF23 were independent predictors of AAC scores.

## 4. Discussion

Our study revealed a high prevalence of AAC in chronic hemodialysis patients, nearly one third of whom, presented with advanced calcification involving four segments. Furthermore, circulating bone-derived biomarker such as sclerostin and FGF23 levels were closely related to the severity of AAC independent of classical risk factors.

It has been generally assumed that CKD patients encounter more distinctive and excessive vascular calcifications, regardless of intimal or medial calcification, which both

contribute to increased arterial stiffness and the high prevalence of sudden cardiac death in dialysis subjects [4,5]. Currently, a number of noninvasive methods have been used for screening and surveillance of vascular calcification, and lumbar lateral plain radiography is one of the simplest techniques with widely feasible follow up. A study by Verbeke et al. on 1084 dialysis patients revealed that semi-quantitative AAC scores, developed from Framingham Heart Study cohorts, could be an independent predictor of cardiovascular events and mortality in dialysis patients [23]. Consistent with the findings of the cohort Calcification Outcome in Renal Disease (CORD) study [24], our result showed that the severity of AAC increased stepwise from L1 to L4. However, the mechanism of aortic calcification extension in a distal to proximal direction remains unknown and need future investigations.

In our study, several demographic factors were identified as independent determinants of AAC, including aging, hypertension, and vascular disease. Furthermore, CRP, a marker of chronic inflammation, was also significantly relevant to vascular calcification. Our previous study had demonstrated a strong relationship between chronic inflammation and the deranged bone mineral metabolism [25]. This finding highlights the important role of chronic inflammation in vascular calcification.

Recently, there is growing evidence of the role of the Wnt-ß-catenin signaling pathway in vascular calcification. Martinez-Moreno et al. reported that activation of the Wnt/β-catenin signaling pathway enhances the phenotype switch and transformation of the vascular smooth

cell into the osteoblast-like cell [10]. Furthermore, sclerostin has been considered as an inhibitor of bone mineralization by suppressing Wnt signaling [8]. In the general population, the circulating sclerostin level was associated with arterial stiffness [12]. However, in CKD patients, the relationship between sclerostin and vascular calcification is inconclusive [14-17]. For hemodialysis patients, Delanaye et al. found no association between sclerostin and the AAC grade [16], whereas Pelletier el al. reported that sclerostin levels correlated with AAC severity [17]. In non-dialysis CKD patients, the high sclerostin level was an independent determinant of absent AAC [15]. Our study revealed the strong association between AAC score and sclerostin, level of sclerostin was independently associated with AAC. The discordance might be attributed to the diversity in the study population and detection methods. One recent study revealed that sclerostin was directly linked to cardiovascular survival in chronic dialysis patients [26]. Our results also revealed a significant association between sclerostin and the biomarkers of bone mineral metabolism. Although the exact mechanism remains unknown, the direct effect of sclerostin on parathyroid hormone activity has been reported [27]. Moreover, whether sclerostin exerts comparable effects on the vascular and skeletal systems, remains largely unknown. Taken together, it appears that the circulating sclerostin level is a useful biomarker of vascular calcification and may have significant prognostic impact on long-term outcomes.

In our study, the DKK-1 level was not associated with vascular calcification in dialysis patients. Despite the nonsignificant relationship between the DKK-1 level and the carotid intima-media thickness in diabetes patients [28], lower serum DKK-1 levels were accompanied by severe AAC in the elderly [29]. Although both the DKK-1 and sclerostin levels similarly act as Wnt pathway inhibitors, it is not surprising that they may exert divergent associations in calcification genesis. There is recent evidence that DKK-1 had less exclusive effects on Wnt-ß-catenin signaling pathway regulation than sclerostin [30] and is not related to renal osteodystrophy in a cross-sectional survey [11]. Progressive increase in blood FGF23 level is a characteristic feature of the CKD population [9]. Irrespective of phosphorous levels, FGF23 independently predicts survival of chronic hemodialysis patients [31,32]. However, whether FGF23 plays an active pathogenetic role in uremic vascular calcification remains uncertain, and experimental studies have exhibited opposing effects on the calcification process [33,34]. In the present study, an inverse association was observed between sclerostin and FGF23. Interestingly, the expression of serum circulating FGF23 level was markedly decreased in sclerostin knockout mice [35]. Further research studies are required in order to examine the interplay between the Wnt pathway and FGF23.

The interpretation of the data from our study is limited by the study design. Caution is necessary since this cross-sectional study only accounted for the associations between the biomarkers and vascular calcification, and did not examine the causal relationship between

these factors. Thus, incident longitudinal prospective studies are needed in order to determine whether sclerostin and FGF23 truly contribute to calcification genesis and are implicated in clinical outcomes. Moreover, the mechanical loading status was not evaluated by accessing daily physical activity, which influences the secretion of bone-derived biomarkers.

## 5. Conclusions

Our study revealed the high prevalence of AAC from the distal to proximal direction and the involvement of multiple segments among uremic patients. Levels of circulating sclerostin, DKK-1, and FGF23 levels all were associated with the AAC severity. There was significant relationship between sclerostin and FGF23 and both were independent determinants of AAC in chronic hemodialysis patients.

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Table 1. Comparison of the clinical characteristics and biochemical data of 227 hemodialysis patients according to AAC severity

| | Total (n=227) | No AAC=0 (n=66) | Mild 1≦AAC≦5 (n=51) | Moderate 6≦AAC≦12 (n=54) | Severe AAC≧13 (n=56) | P value |
|---|---|---|---|---|---|---|
| Calcification score | 5 (0-12) | 0 (0-0) | 3 (2-4) | 8 (6-10) | 17 (14-21) | <0.0001 |
| Age | 63.0±10.1 | 58.5±8.4 | 62.2±1.1 | 65.4±9.9 | 66.9±8.9 | <0.0001 |
| Gender, male (%) | 103 (45) | 23 (35) | 25 (49) | 24 (44) | 31 (55) | NS |
| BMI | 23.1±3.7 | 22.5±3.6 | 23.6±3.3 | 22.7±3.7 | 24.6±3.7 | 0.044 |
| Duration (years) | 8.6±5.8 | 9.3±5.8 | 7.4±6.1 | 9.2±6.0 | 8.4±5.5 | NS |
| Kt/V | 1.58±0.20 | 1.55±0.25 | 1.62±0.18 | 1.57±0.21 | 1.59±0.16 | NS |
| DM (%) | 78(34) | 15(23) | 17(33) | 24(44) | 22(39) | NS |
| HTN (%) | 161(71) | 38(58) | 35(69) | 44(82) | 44(79) | 0.016 |
| Vascular disease (%) | 70(31) | 8(12) | 13(25) | 23(43) | 26(46) | <0.0001 |
| Calcium (mg/dL) | 9.5±0.8 | 9.5±0.7 | 9.4±0.8 | 9.3±0.8 | 9.7±0.8 | 0.018 |
| Phosphorous (mg/dL) | 4.8±1.2 | 4.8±1.1 | 4.8±1.2 | 4.4±1.2 | 4.9±1.2 | NS |
| Ca X P ($mg^2/dL^2$) | 45.1±11.9 | 45.7±11.0 | 45.5±11.6 | 41.2±11.8 | 47.8±12.7 | 0.029 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Intact PTH (pg/ml) | 228 (83-491) | 224.5 (105.5-520.5) | 183.0(56.0-417.0) | 173.5 (97.3-349.3) | 342.5 (69.0-653.5) | NS |
| Albumin (mg/dL) | 4.0±0.3 | 4.0±0.3 | 4.0±0.2 | 4.0±0.4 | 3.9±0.3 | NS |
| Alk-P (mg/dL) | 85.7±38.3 | 85.2±39.0 | 78.0±39.9 | 93.3±50.2 | 86.1±29.5 | NS |
| hsCRP (mg/dL) | 2.90 (1.30-6.22) | 2.67 (1.22-4.98) | 2.15 (1.06-4.90) | 2.95 (1.31-5.53) | 3.85 (1.68-10.3) | 0.032 |
| Total cholesterol (mg/dL) | 162.3±34.8 | 163.4±36.4 | 164.5±36.8 | 159.2±28.4 | 161.9±37.1 | NS |
| LDL (mg/dL) | 86.0±28.6 | 85.1±31.9 | 86.4±28.5 | 83.5±21.7 | 89.0±39.0 | NS |
| Sclerostin (pg/ml) | 619.4 (452.0-789.9) | 641.1(492.9-798.5) | 640.4 (524.5-908.2) | 612.6 (411.9-768.9) | 561.8 (385.7-738.3) | 0.034 |
| DKK-1 (pg/ml) | 313.1(208.2-435.8) | 353.9(244.7-495.1) | 313.1 (223.7-448.7) | 277.2(201.5-380.0) | 274.1(181.0-423.5) | NS |
| FGF23 (pg/ml) | 3131 (496-14004) | 2775.0(569.0-11832.0) | 2243.1(443.0-12684.3) | 1765.0 (247.0-8130.0) | 9461.5(1535.3-26584.8) | 0.011 |

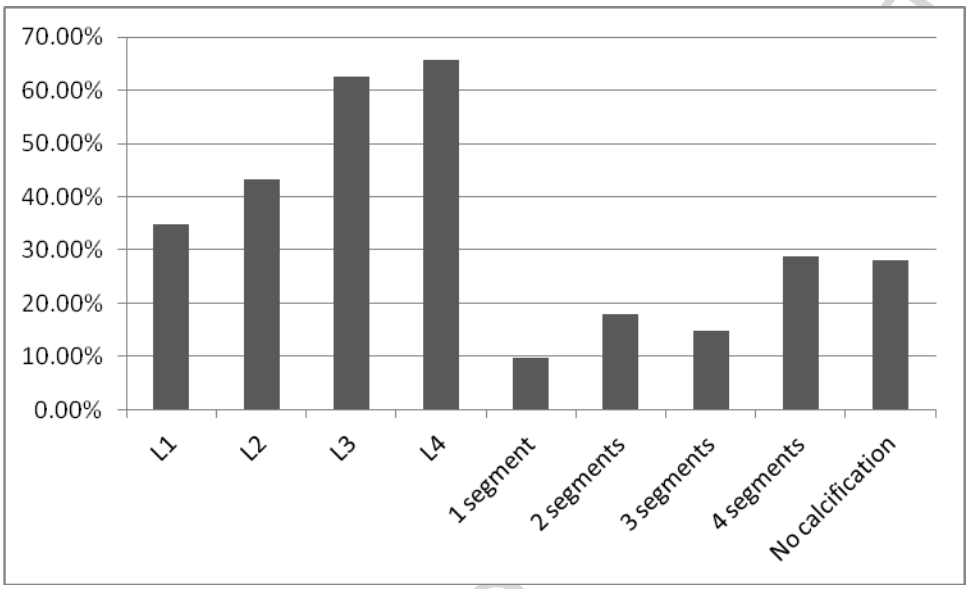
AAC: abdominal aortic calcification; BMI: body mass index; Ca X P: calcium phosphate product; hsCRP: high-sensitivity C-reactive protein; LDL: low density lipoprotein; DKK-1: Dickkopf-1; FGF23: fibroblast growth factor 23

Table 2. Factors associated with AAC scores in hemodialysis patients

| | ß | 95% CI | P value |
|---|---|---|---|
| Age | 0.185 | 0.093 to 0.276 | <0.0001 |
| BMI | 0.035 | -0.209 to 0.278 | NS7 |
| Diabetes | 1.326 | -0.651 to 3.303 | NS |
| Hypertension | 2.407 | 0.414 to 4.399 | 0.014 |
| Vascular disease | 2.543 | 0.583 to 4.504 | 0.016 |
| Calcium | 0.457 | -0.880 to 1.794 | NS |
| Ca x P | -0.050 | -0.138 to 0.037 | NS |
| Albumin | -2.386 | -5.493 to 0.720 | NS |
| hsCRP | 1.948 | 0.232 to 3.665 | 0.026 |
| Sclerostin | -5.718 | -10.735 to -0.701 | 0.026 |
| DKK-1 | -3.374 | -6.974 to 0.226 | NS |
| FGF23 | 1.940 | 0. 614 to 3.267 | 0.004 |

AAC: abdominal aortic calcification; CI: confidence interval; BMI: body mass index; Ca x P: calcium phosphate product; hsCRP: high-sensitivity C-reactive protein; DKK-1: Dickkopf-1; FGF23: fibroblast growth factor 23

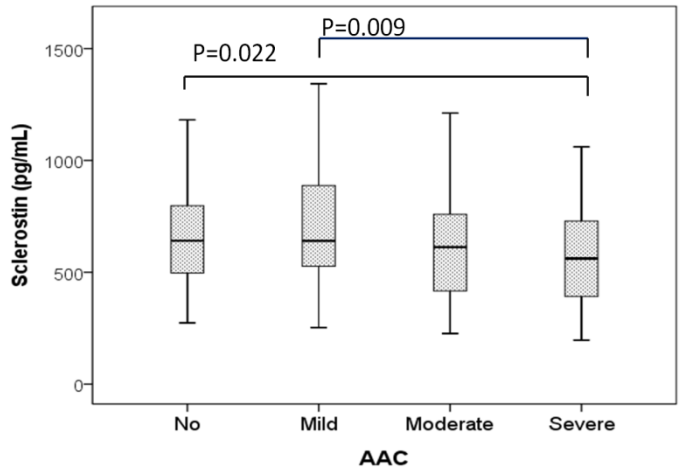
Fig.1. Distribution of AAC lesions according to the number and location of the involved segments



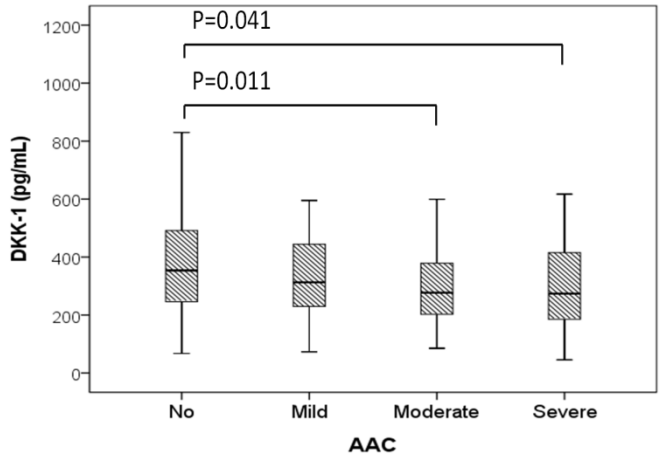

AAC : abdominal aortic calcification

Fig.2. The relationship between bone-derived biomarkers and the severity of abdominal aortic calcification (AAC). (a) sclerostin; (b) DKK-1; (c) FGF23

(a)




(b)



(c)

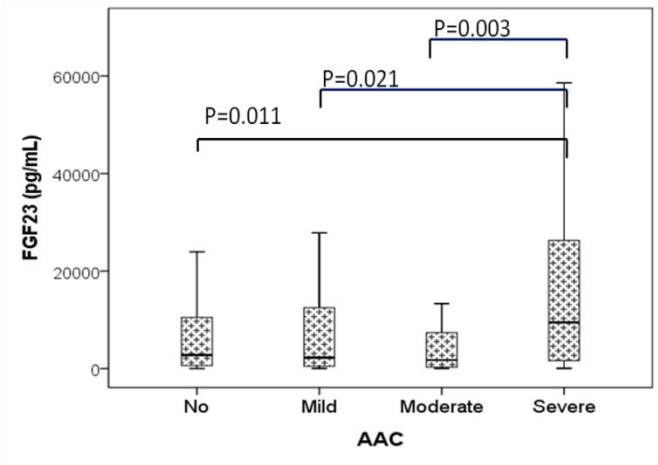
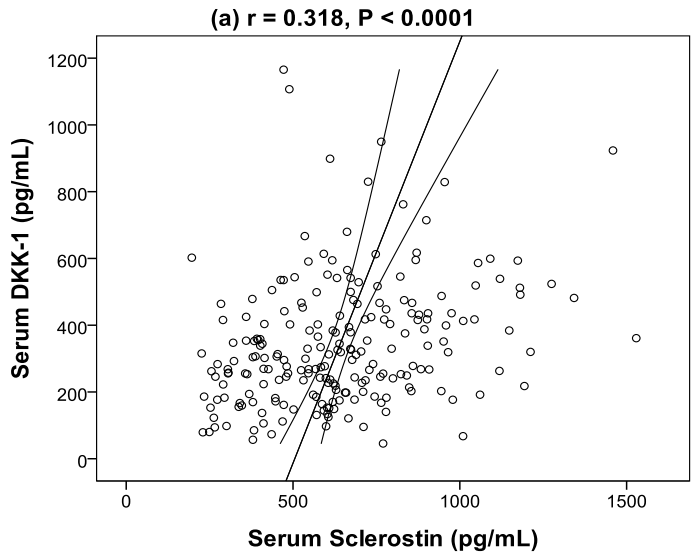
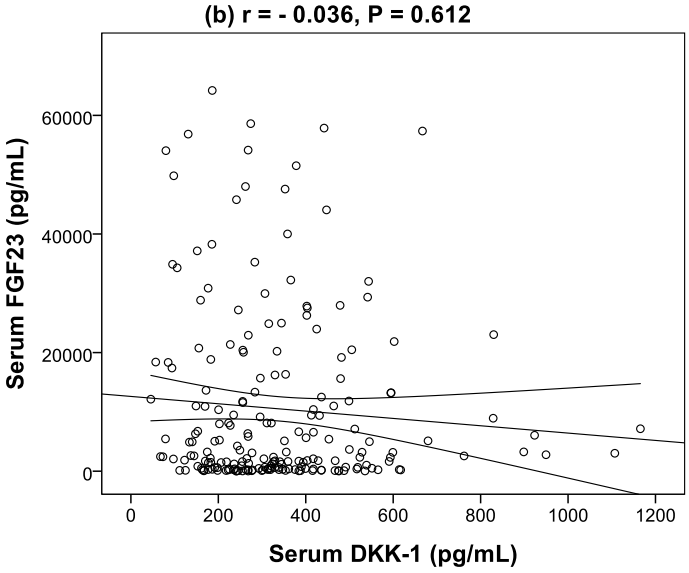



Fig.3. The relationship between bone-derived biomarkers (a) sclerostin and DKK-1; (b) DKK-1 and FGF23; and (c) sclerostin and FGF23





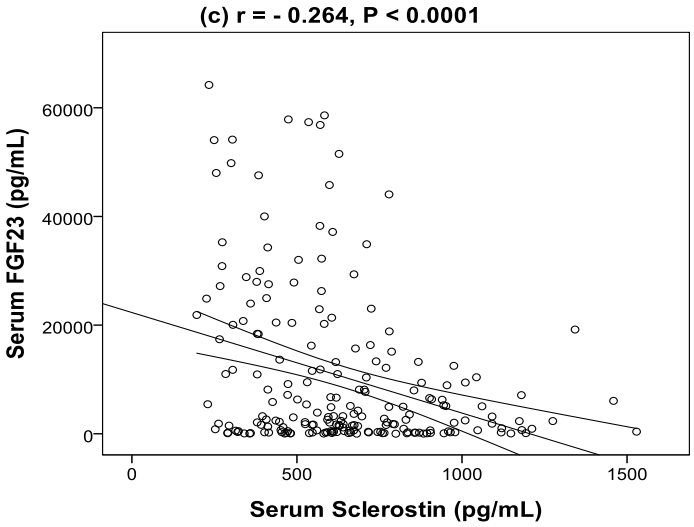
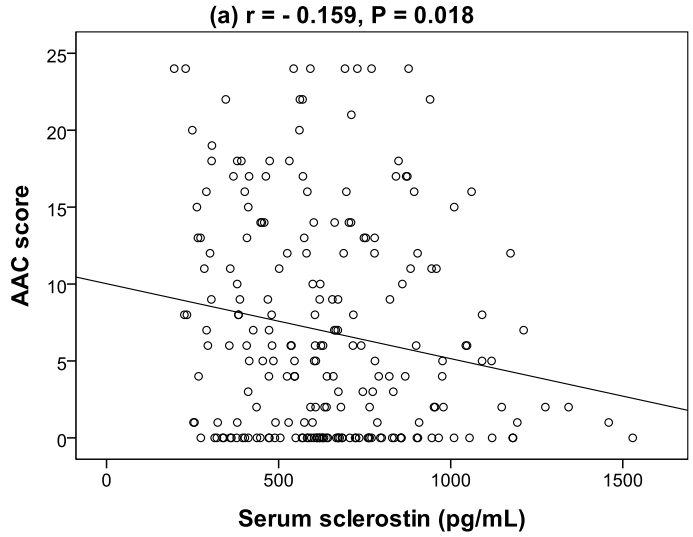
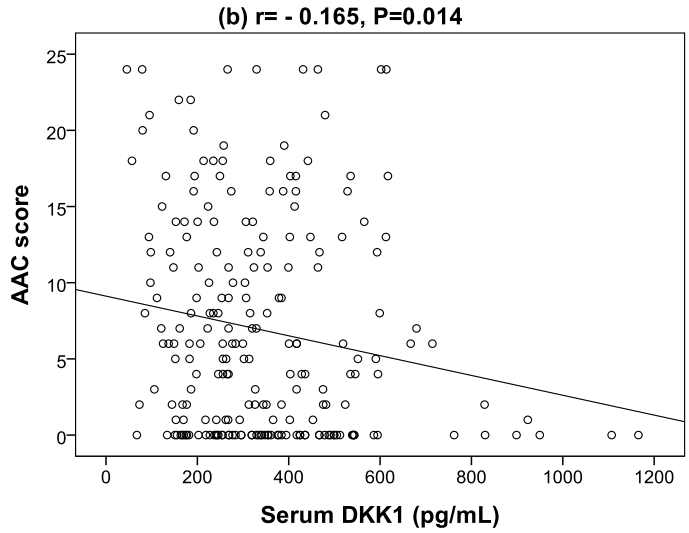
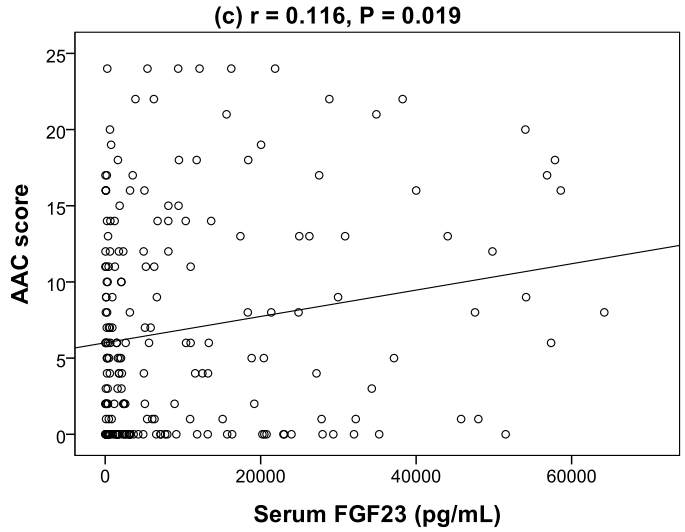



Fig.4. The relationship between abdominal aortic calcification (AAC) scores and bone-derived biomarkers





1. The prevalence of vascular calcification assessed by lateral film X-ray of lumbar spine was 71% in chronic hemodialysis patients.
2. Levels of circulating sclerostin, DKK-1 and FGF23 were directly related to severity of vascular calcification.
3. Sclerostin positively correlated with DKK-1 but was inversely associated with FGF23.
4. In addition to traditional factors, bone-derived biomarkers: sclerostin and FGF23 were independent determinants of vascular calcification.