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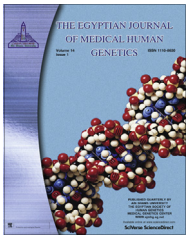

ORIGINAL ARTICLE

Association of serum fetuin-A and fetuin-A gene polymorphism in relation to mineral and bone disorders in patients with chronic kidney disease

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Abstract Disorders of bone and mineral metabolism contribute to an increased prevalence of vascular calcification (VC) with its adverse clinical outcomes in patients with chronic kidney disease (CKD). The pathogenesis of VC is not fully understood. Fetuin-A is one of the inhibitors of calcification whose level is lowered in patients with CKD. In addition fetuin-A 256Ser/Ser (allele G) might affect serum fetuin-A levels. The aim of this work was to study the association between

Abbreviations: CKD, chronic kidney diseases, VC, vascular calcification, HD, hemodialysis, BMD, bone mineral density, CRP, C-reactive protein, e-GFR, estimated glomerular filteration rate, Ca×PO4, calcium by phosphorus product, iPTH, intact parathyroid hormone, Alk, alkaline phosphatase, ESRD, end stage renal disease, CVD, cardiovascular disease, VSMCs, vascular smooth muscle cells, EBCT, electron beam computed tomography, CKD–MBD, chronic kidney disease–mineral and bone disorder, IMD, intima-media thickness, RTRs, renal transplant recipients, RT, renal transplantation, CACs, coronary artery calcification score

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fetuin-A and its gene and VC and also with bone mineral density (BMD) in patients with CKD on conservative treatment, on maintenance of hemodialysis (HD) and those who underwent renal transplantation.

This study included twenty eight CKD patients on HD, seventeen CKD patients on conservative treatment and twelve patients who underwent transplantation in addition to sixteen healthy controls. All were subjected to history taking, clinical examination, laboratory investigations including fasting serum glucose, urea, creatinine, albumin, lipid profile, C-reactive protein (CRP), estimated glomerular filteration rate (e-GFR), calcium, phosphorus, calcium by phosphorus product (Ca×PO4), intact parathyroid hormone (iPTH), alkaline phosphatase (Alk), fetuin-A and genotyping for the common functional polymorphisms on fetuin-A (Thr256Ser) using the Polymerase chain reaction (PCR) technique. Radiological examination included ultrasonography of carotid arteries and assessment of VC by plain X-ray and assessment of BMD.

Serum calcium was lower, phosphorus, Ca×PO4, iPTH and Alk were higher in all patient groups than control. Fetuin-A was lower in all patient groups compared to controls. VC was detected in 39.2% HD patients, 29.4% patients on conservative treatment and 25% patients on transplantation. T-score of BMD was significantly lower in all patient groups than control. There was no statistically significant difference between patients and control groups according to the frequencies of the three fetuin-A genotypes (C → G) but the distribution of the fetuin-A (C → G); Thr256Ser gene polymorphisms in the studied subjects showed significant correlation with low serum fetuin-A levels. VC was associated with older age, male gender, longer HD duration, lower albumin, higher LDL-c, higher carotid plaques and lower T-score value of BMD.

VC was evident in patients with CKD and it is related to atherosclerosis and lower BMD. Fetuin-A was lower in all patients with CKD with significant relation between serum fetuin-A level and its gene polymorphism but not with VC.

1. Introduction

Chronic kidney disease (CKD) is associated with increasing risk for cardiovascular disease (CVD) and cardiovascular events, even a slight reduction in glomerular filtration rate (GFR) is associated with a significant increase in cardiovascular risk [1,2]. Up to 45% of pre-dialysis CKD patients may die before reaching end-stage renal disease (ESRD), with CVD being the leading cause of death with increasing risk in ESRD patients [3,4].

Despite an improvement in survival after renal transplantation, renal transplant recipients (RTRs) remain at an increased risk for CVD [5].

Vascular calcification (VC) is a strong independent risk factor for CV mortality [6]. VC may promote arterial stiffness, which leads to increased pulse pressure, left ventricular hypertrophy and cardiovascular events [7,8].

The pathogenesis of VC is complex and not fully understood. Numerous risk factors have been reported, some of these are classic such as ageing, hypertension, diabetes, smoking and dyslipidemia [9,10]; certain others are more specific to CKD, such as mineral metabolism abnormalities, particularly hyperphosphatemia, extreme parathyroid hormone, serum levels or excess administration of calcium salts and dialysis duration [10,11].

There are two main types of VC, intimal and medial calcifications (Monckeberg's medial sclerosis). Both types of calcifications are present in patients with CKD, but the complications of these two types of VC are different: The former is related to calcification of the atherosclerotic plaque and is mainly associated with occlusion of the vessels, and the latter is associated with vascular stiffness; both are responsible for the increased mortality in patients with CKD [12].

VC is a pathological process occurring in response to an inappropriate environmental milieu [13]. It does not consist of a simple precipitation of calcium and phosphate but is instead an active and modifiable process in which, step by step, the vascular smooth cells undergo apoptosis and vesicle formation changes the phenotype of smooth vascular cells into osteoblast like cells capable of undergoing the mineralization process [12,14].

There are multiple inhibitors of VC such as matrix GLA protein (MGP), osteoprotegerin (OPG), pyrophosphates and fetuin-A [15,16]. Animal knockout models have shown that key gene products may be protective of VC including MGP, OPG and fetuin-A [17–19].

Fetuin-A is a hepatically synthesized 62-kDa {also called alpha2-Heremans Schmid glycoprotein (AHSG)} and a negative acute phase reactant; it is a member of the cystatin superfamily of cysteine protease inhibitors [20,21]. It is a carrier for growth factors, binds to and inactivates transforming growth factor (TGF)-β and bone morphogenic protein, and is a major component of mineralized bone. It decreases macrophage activation and release of proinflammatory cytokines [22,23]. Fetuin-A interacts with small mineral nuclei to form soluble colloidal particles thereby preventing the precipitation of basic calcium phosphate (BCP) and acting as a calcification inhibitor in the supersaturated extracellular environment [24]. Consequentially, fetuin-A can inhibit calcification in circulation without affecting the normal bone mineralization [25]. In addition, at the cellular level, fetuin-A inhibits apoptosis of vascular smooth muscle cells (VSMCs), it also aids in the binding of apoptotic bodies to neighboring cells leading to their clearance and thereby reducing the potential for these substances to nucleate BCP [26,27]. Fetuin-A is also known to inhibit the insulin stimulated tyrosine kinase receptor and has been shown

to be associated with metabolic syndrome and an independent risk factor for the development of type 2 diabetes mellitus [28,29].

In CKD patients, serum level of fetuin-A is significantly lower than in healthy control subjects [30]. Specific polymorphisms of fetuin-A gene were demonstrated to influence circulating levels of fetuin-A [31]. However others concluded that the distribution of the fetuin-A (C → G); gene polymorphisms does not affect serum fetuin-A levels [32].

Ketteler et al. demonstrated a relationship between all cause/cardiovascular mortality and low serum fetuin-A in HD patients [33]. Some studies permit consideration of fetuin-A as the missing link between inflammation, atherosclerosis and calcification in patients with CKD, extending the previously known term "malnutrition-inflammation-atherosclerosis syndrome" to "malnutrition-inflammation-atherosclerosis-calcification syndrome" [34,35]. Hernandez et al. considered that the presence of preexisting VCs may be associated with an increased cardiovascular risk after renal transplantation [36].

Patients with CKD show VCs almost in all localizations, from high caliber arteries such as aorta, where the prevalence is extremely high, to medium and small sized vessels, including coronary arteries. Also calcification of the cardiac valves represents a high risk for cardiovascular dysfunction [37].

VCs can be evaluated by different methods: ultrasonography, electron beam computed tomography (EBCT), multislice computed tomography or plain X-ray. Although the plain X-ray approach might be relatively crude but it has the advantages of being simple, cheap and applicable in daily clinical practice [13,38,39].

Low bone mineral content at menopause has been shown to be a risk factor for increased cardiovascular mortality later in life [40]. Patients with severe VCs were associated with a higher prevalence of any osteoporotic fracture. In addition, the progression of the aortic vascular calcifications was associated with the rate of decline in BMD at the lumbar spine [41]. Low bone density has also been associated with aortic calcification, as detected by conventional X-ray and with surrogate markers of subclinical vascular disease [42]. An association between VC, vertebral fractures and mortality has been found in patients on HD [43].

The term chronic kidney disease–mineral and bone disorder (CKD–MBD) has been applied to the disturbance of mineral metabolism, renal bone disease and VC, together with patient level outcomes of fracture, CVD and mortality in patients with CKD [44].

2. Aim of the work

The aim of this work was to study the association between serum fetuin-A level and its gene and vascular calcification as well as with bone mineral density in patients with CKD on conservative treatment, on maintenance HD and those who underwent renal transplantation.

3. Subjects and methods

The present study included four groups of subjects: Patients with chronic kidney disease were selected from nephrology wards, outpatients clinics and the hemodialysis unit at the Nephrology Department, Medical Research Institute, Alexandria University, Egypt. The study protocol was conducted according to the Helsinki Declaration. Informed consent was obtained from all the recruited subjects prior to their inclusion in the study and approved by the Clinical Research Ethics Committee of the Medical Research Institute.

Patients were divided into three groups. Twenty eight patients were receiving 4 h HD sessions, three times weekly for more than 6 months, with bicarbonate dialysate using polysulphon dialyzer (Group I), seventeen patients were on conservative treatment (stages 3,4) (Group II), twelve patients underwent transplantation (Group III), all of the kidney transplant recipients were on a triple immunosuppression regimen consisting of prednisolone, cyclosporine and mycophenolate mofetil or azathioprine.

Subjects underwent a 2-week washout period, in which all phosphate binders were withheld. None of the patients had ever been treated with bisphosphonates.

Sixteen healthy volunteers were taken as control group (Group IV).

Patients with diabetes mellitus, liver disease, active infection or overt inflammation and malignancies were excluded from the study.

The studied groups were subjected to the following:

I- **Detailed medical history taking**; stressing on bone aches and history of fracture and clinical examination was performed after taking an informed consent from each subject.

II- **Calculation of body mass index (BMI)** (weight in kilograms/height in meters squared) [45].

III- **Laboratory investigations**:

(A) **Specimen collection, storage and preparation**: Five milliliters of venous blood were withdrawn from every patient and control in the morning after over night fasting. 500 μl whole blood was separated in an eppendorf containing ethylenediamine tetraacetic acid (EDTA) 5% and stored at −20 °C for genotyping for the common functional polymorphisms on fetuin-A (Thr256Ser) using Polymerase chain reaction (PCR) technique. The remaining sample was transferred into a disposable plastic tube and allowed to clot, then was centrifuged at 1200 rpm for 10–15 min to separate the serum. The serum was then divided into three aliquots which were kept frozen at −20 °C until use. The following laboratory investigations were carried out:

(B) **Routine laboratory investigations**: They included estimation of fasting serum glucose, urea, creatinine, triglycerides, total cholesterol and high-density lipoprotein (HDL) cholesterol using enzymatic colorimetric methods. Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald's formula. Also, total calcium, phosphorus, alkaline phosphatase and serum albumin were measured. Determination of routine biochemical parameters was performed with standard techniques by using the auto-analyzer OLYMPUS AU400, using reconstituted freeze dried forms of multi-analyte calibrators for the serum samples [46,47].

(C) **Intact parathyroid-hormone (i-PTH)** was measured by chemiluminescent immunometric assay on IMMULITE 1000 autoanalyzer [48].

(D) **Estimation of the glomerular filteration rate (GFR)** from the plasma creatinine with the simplified Modification of diet in renal disease (MDRD) formula: GFR (ml/min/1.73 m^2 body surface area) = 175 × (serum creatinine, in mg/dl)$^{-1.154}$ × (age)$^{-0.203}$ × (0.742, if female) or × (1.212, if African American) [49].

(E) **Specific laboratory tests**:

1. **Quantitative analysis of fetuin-A**: Serum fetuin-A level was measured using the ELISA technique (BioVendor Human Fetuin-A ELISA,Germany) following the instructions of the manufacturer [50].
2. **DNA extraction and genotyping for the common functional polymorphisms on fetuin-A (Thr256Ser)** using the PCR technique [51].

DNA was extracted from peripheral blood leucocytes from the freshly withdrawn whole blood, using GeneJET™ Genomic DNA Purification Kit, Fermentas. [(i)]The purity and concentration of extracted genomic DNA were determined using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific,Wilmington, Delaware USA). Polymerase chain reaction and Restriction fragment length polymorphism (PCR/RFLP) techniques were used to detect fetuin-A gene (Thr256Ser) polymorphism. PCR amplification of extracted DNA was done in a total volume of 25 µl (5 µl extracted DNA was mixed with 1 µl of each forward and reverse primer and 12.5 µl DreamTaq™ Green PCR Master Mix (2×) and completed to 25 µl with nuclease-free water).The primers used (purchased from Fermentas Life Sciences.) were: F5-GTCACCCCTCCTTGTAAC-3 (sense)[Tm = 45.6 °C] Reverse primer antisense: R5-CCCCAATGAGACCACA-3 (Antisense) [Tm = 46 °C] PCR was done on the thermal cycler (Biometra) and the reaction conditions were as follows: initial denaturation at 95 °C for 5 min then 35 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 15 min.

For mutational analysis of fetuin-A Thr256Ser (c.766C > G) at Sac I recognition site; FastDigest® SacI restriction enzyme 100 µl (from Fermentas Life Sciences) was used. 10 µl of each PCR product was mixed with FastDigest® enzyme 1 µl with FastDigest® buffer 2 µl and completed to a total volume of 30 µl with nuclease-free water. They were mixed gently and incubated at 37 °C for 60 min.

The digested products were separated on 1.5% agarose gel. Allele C does not contain the SacI site, remains undigested as 405 bp fragments, whereas the allele G yields 193- and 212-bp fragments. There are three possible genotypes for the fetuin-A: Thr/Thr (Allele C), Thr/Ser (heterozygote) and Ser/Ser (Allele G); (homozygote for absence of the Sac I site, heterozygote and homozygote for the presence of the Sac I site; respectively) [51] (Fig. 1).

IV. **Radiological investigations**:

1- Carotid ultrasonography for the measurement of intima-media thickness (IMT) and the presence of atherosclerotic plaques [52].

2- Vascular calcification (VC) was evaluated by a simple vascular calcification score (SVCS) which is based on the analysis of plain radiographic films of the pelvis and hand. Pelvis films were divided into four sections by two imaginary lines: a horizontal line over the upper limit of both femoral heads and a median vertical line over the vertebral column. Hand films were divided for each hand by a horizontal line over the upper limit of the metacarpal bones. Pelvis films evaluated iliac and femoral arteries (ileo-femoral score) and hand films evaluated radial and digital arteries (hand score). The presence of vascular calcifications in each section was rated as 1 and its absence as 0. The final score was the sum of all sections and ranged from 0 to 8 [53].

3- The dual X-ray absorptiometry device to assess bone mineral density (BMD), was calculated at the lumbar spine and the femoral neck (g/cm^2), it was expressed in standard deviation (SD) units as T-scores (comparison with the mean bone density in young adults) and *Z*-scores (comparison with age matched mean). According to the World Health Organization, BMD in any adult that is between 1 and 2.5 SD below the mean is defined as osteopenia, and a value more than 2.5 SD below the mean is defined as osteoporosis [54].

4. Statistical analysis

Study data were statistically analyzed using the Statistical Package for Social version program (SPSS program-version 18.0 – SPSS Inc., Chicago, IL. USA). Quantitative data were expressed as median, minimum and maximum as well as mean ± Standard deviation (SD). Patients were studied as a group, and successively divided into three groups. Qualitative data were described using number and percent. Association between categorical variables was tested using the Chi-square test. When more than 20% of the cells have an expected count less than 5, correction for chi-square was conducted using the Firsher's Exact test or the Monte Carlo correction.

The distributions of quantitative variables were tested for normality using Kolmogorov–Smirnov's test, Shapiro–Wilk's test. D'Agstino test was used if there was a conflict between the two previous tests. If they reveal normal data distribution, parametric tests were applied. If the data were abnormally distributed, non-parametric tests were used.

For normally distributed data, comparison between two independent populations was done using the independent *t*-test while more than two populations were analyzed by the *F*-test (ANOVA) and the Post Hoc test (LSD).

For abnormally distributed data, Mann–Whitney's Test (for data distribution that was significantly deviated from normal) was used to analyze two independent populations. If more than two populations were analyzed, the Wallis test was used.

Correlations between variables were assessed using the Spearman or Pearson's correlation coefficient. A *P* value of less than 0.05 was judged to be significant.

5. Results

The present study included CKD patients, twenty eight on HD, seventeen on conservative treatment, and twelve transplanted patients with sixteen subjects as the control group. They were of matched age and sex (Table 1).

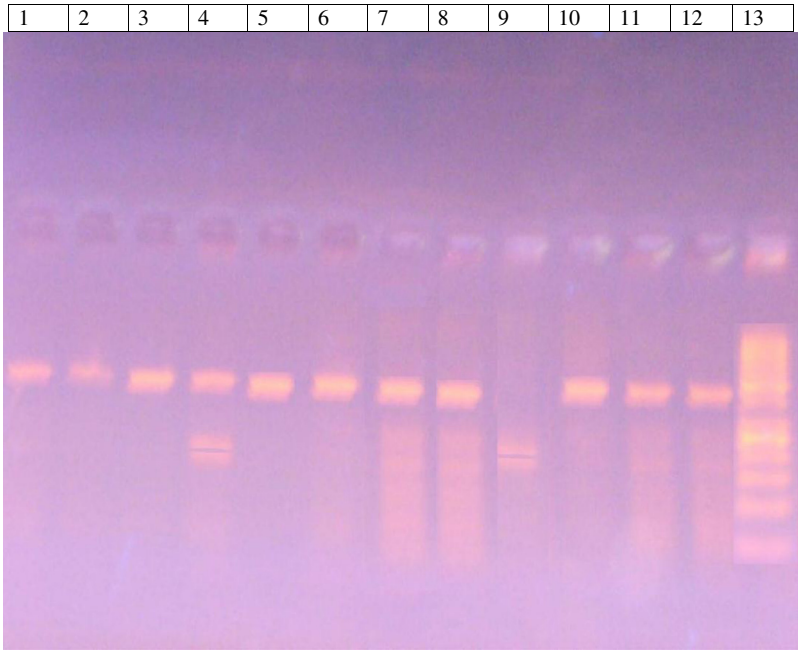

Figure 1 Ethidium bromide stained agarose gel electrophoresis of PCR amplified fetuin-A gene(C → G); Thr256Ser following sac I restriction digestion for some samples. Showing: Lane 13: DNA size marker 50–1000 bp. Lane 9: Allele G (Ser/Ser) homozygote for the presence of the sacI site which digests the 405 bp fragment into 193 and 212 bp fragments in a homozygote patient. Lane 1,2,3,5,6,7,8,10,11,12: Allele C (Thr/Thr) undigested as 405 bp. fragments homozygote for absence of the sacI site. Lane4: heterozygote patient (Thr/Ser).

Etiology of CRF on HD patients was variable, in 15 patients (53.5%) it was hypertension, in 3 patients (10.7%) it was chronic pyelonephritis, in 1 patient (3.6%) it was analgesic nephropathy, in 1 patient (3.6%) it was the nephritic syndrome, in 1 patient (3.6%) it was polycystic kidney, in 1 patient (3.6%) it was renal amyloidosis, in 1 patient (3.6%) it was congenital and in 5 patients (17.9%) it was of unknown etiology.

Twenty one patients on HD (75%) were complaining of bone ache with a history of fracture in one patient only (3.6%); ten patients on conservative treatment (58.8%) and only three patients in the transplanted group (25%) were complaining of bone ache.

In the HD group, fifteen patients (53.6%) were hypertensive; ten patients on conservative treatment (58.8%) were hypertensive and three patients in the transplanted group (25%) were hypertensive. In the HD group, seven patients (25%) had clinical manifestations and ECG changes of IHD; in those on conservative treatment, five patients had IHD (29.4%) while in the transplanted group, only two patients (16.7%) had IHD.

Duration of HD was 63.46 ± 54.14 months while the duration of transplantation was 31.17 ± 11.17 months.

Serum albumin was significantly lower in groups I, II and III patients than in the controls (Table 2).

Total cholesterol and LDL-c were significantly higher in group III than in the control group, also LDL-c was significantly higher in group I and II than the control group. HDL-c was significantly lower in group I and II than in the control group and TG was significantly higher in group III than the control group. CRP was significantly higher in group I and II but not in group III than in the control group. e-GFR was significantly lower in all patient groups than in the control group (Table 3).

Serum calcium was significantly lower in all patient groups than in the control group, serum phosphorus and Ca×PO4, iPTH, and Alk were significantly higher in all patient groups than in the control group. Serum fetuin-A was significantly lower in groups I, II and III than in the control group (Table 4).

IMT was significantly higher in all patient groups than in the control group. Plaques were detected in eight patients (28.6%) on HD, in four patients (23.5%) on conservative treatment and in only two patients (16.7%) who underwent transplantation. (Fig. 2). l Vascular calcification was detected in eleven HD patients (39.3%), five patients on conservative treatment (29.4%) and three transplanted patients (25%) (Table 5).

As regards BMD of the arm, it was significantly lower in group I, II and III than in the control group but reach a significant value only in group III ($P_2 = 0.023^*$). BMD of the pelvis was lower in all patient groups than in the control group but was of significant value in groups I and III only ($P = 0.048^*$, $P_2 = 0.026^*$ respectively).BMD of the lumbar spine was lower in all patient groups than in the control group but of significant value in group I only ($P = 0.0016^*$). T-score was significantly lower in all patient groups than in the control group and the *Z*-score was significantly lower in group I and III ($P < 0.001^*$, $P_2 < 0.001^*$ respectively) (Table 6).

Table 1 Demographic data of ESRD patients and control groups.

	Group I (n = 28)	Group II (n = 17)	Group III (n = 12)	Group IV (n = 16)	Test of sig.	P value
Sex						
Male	11 (39.3%)	8 (47.1%)	4 (33.3%)	7 (43.8%)	χ^2 = 0.084	P = 0.772
Female	17 (60.7%)	9 (52.9%)	8 (66.7%)	9 (56.3%)	χ^2 = 0.036 FEp	P_1 = 0.849 P_2 = 0.705
Age (years)						
Min–Max	21.0–75.0	25.0–80.0	46.0–60.0	27.0–55.0	t = 0.354	P = 0.725
Mean ± SD	43.86 ± 11.59	50.71 ± 13.57	52.25 ± 4.39	42.69 ± 8.32	t = 2.030	P_1 = 0.051
Median	41.0	54.0	52.0	43.50	t = 3.927	P_2 = 0.001*
BMI (kg/m)						
Min–Max	12.03–36.50	22.10–41.90	21.10–34.70	21.90–28.10	t = 0.530	P = 0.599
Mean ± SD	23.65 ± 5.93	28.09 ± 4.67	27.49 ± 3.70	24.29 ± 1.90	t = 3.024	P_1 = 0.005*
Median	23.0	27.30	27.90	24.05	t = 2.976	P_2 = 0.006*
MBP (mmHg)						
Min–Max	83.30–116.60	80.0–123.30	90.0–118.30	80.0 ± 93.30	t = 6.005	P = 0.000*
Mean ± SD	101.39 ± 8.39	107.33 ± 14.19	98.99 ± 8.87	87.39 ± 5.33	t = 5.402	P_1 = 0.000*
Median	103.30	113.30	94.95	89.30	t = 4.314	P_2 = 0.003*
SBP (mmHg)						
Min–Max	110.0–160.0	100.0–170.0	120.0–155.0	100.0–125.0	t = 4.809	P = 0.000*
Mean ± SD	132.50 ± 13.51	141.47 ± 20.52	130.42 ± 11.96	114.06 ± 9.53	t = 4.967	P_1 = 0.000*
Median	130.0	150.0	127.50	117.50	t = 4.314	P_2 = 0.001*

χ2: Chi square test; t: Student's t-test.
FE: Fisher's exact test.
Group I = Patients on HD; Group II = Patients on conservative treatment.
Group III = Patients after transplantation; Group IV = Control group.
ESRD = End stage renal disease; BMI = Body mass index; MBP = Mean blood pressure; SBP = Systolic blood pressure.
P = Comparison between control and group I; P_1 = Comparison between control and group II.
P_2 = Comparison between control and group III.
* Statistically significant at $P \leqslant 0.05$.

Table 2 Laboratory investigations in patient and control groups.

	Group I (n = 28)	Group II (n = 17)	Group III (n = 12)	Group IV (n = 16)	Test of sig.	P value
FBS (mg/dl)						
Min–Max	76.0–111.0	89.0–109.0	83.0–102.0	70.0–92.0	t = 4.153	P < 0.001*
Mean ± SD	92.79 ± 12.07	98.53 ± 6.38	94.58 ± 6.40	81.56 ± 5.80	t = 7.976	P_1 < 0.001*
Median	90.0	99.0	96.0	82.50	t = 5.626	P_2 < 0.001*
Creatinine (mg/dl)						
Min–Max	3.60–16.50	4.0–8.10	1.0–1.90	0.80–1.0	Z = 5.235	P < 0.001*
Mean ± SD	8.46 ± 3.27	5.20 ± 1.38	1.48 ± 0.29	0.86 ± 0.06	Z = 3.803	P_1 < 0.001*
Median	8.10	4.80	1.45	0.90	Z = 0.883	P_2 < 0.377
Urea (mg/dl)						
Min–Max	80.0–207.0	85.0–140.0	30.0–108.0	19.0–40.0	t = 17.411	P < 0.001*
Mean ± SD	139.04 ± 32.80	106.65 ± 15.92	64.25 ± 25.02	28.31 ± 5.69	t = 19.040	P_1 < 0.001*
Median	145.0	102.0	60.0	28.50	t = 4.883	P_2 < 0.001*
Albumin (mg/dl)						
Min–Max	1.30–4.50	2.60–4.30	3.0–4.30	3.60–4.80	t = 5.632	P < 0.001*
Mean ± SD	3.26 ± 0.75	3.38 ± 0.53	3.66 ± 0.41	4.17 ± 0.31	t = 5.253	P_1 < 0.001*
Median	3.25	3.20	3.70	4.15	t = 3.727	P_2 < 0.001*

t: Student's t-test; Z: Mann Whitney's test.
FBS = Fasting blood sugar.
Group I = Patients on HD; Group II = Patients on conservative treatment.
Group III = Patients after transplantation; Group IV = Control group.
P = Comparison between control and group I; P_1 = Comparison between control and group II.
P_2 = Comparison between control and group III.
* Statistically significant at $P \leqslant 0.05$.

Table 3 Lipid profile, CRP and e-GFR in patient and control groups.

	Group I (n = 28)	Group II (n = 17)	Group III (n = 12)	Group IV (n = 16)	Test of sig.	P value
T.Cholesterol (mg/dl)						
Min–Max	103.0–259.0	115.0–254.0	176.0–203.0	99.20–189.90	Z = 0.171	P = 0.864
Mean ± SD	163.43 ± 43.07	165.47 ± 38.21	190.58 ± 9.08	154.60 ± 22.55	Z = 0.613	P_1 = 0.540
Median	150.50	160.0	190.0	158.50	Z = 4.137	P_2 < 0.001*
LDL-c (mg/dl)						
Min–Max	59.0–171.0	55.0–202.0	109.0–135.0	34.44–105.40	t = 2.538	P = 0.015*
Mean ± SD	105.29 ± 32.01	111.00 ± 42.73	121.50 ± 8.70	82.53 ± 21.13	t = 2.447	P_1 = 0.022*
Median	98.0	105.0	122.0	91.42	t = 6.660	P_2 = 0.001*
HDL-c(mg/dl)						
Min–Max	13.0–42.0	22.0–57.0	42.0–67.0	32.50–71.0	Z = 4.041	P < 0.001*
Mean ± SD	34.68 ± 12.96	35.18 ± 11.71	53.08 ± 7.27	53.04 ± 10.73	Z = 3.861	P_1 < 0.001*
Median	31.0	30.0	53.0	54.90	Z = 0.628	P_2 = 0.530
TG (mg/dl)						
Min–Max	54.0–234.0	45.0–225.0	85.0–250.0	63.0–141.0	Z = 0.854	P = 0.393
Mean ± SD	117.75 ± 50.77	119.06 ± 53.54	185.08 ± 42.57	98.11 ± 2.85	Z = 0.955	P_1 = 0.339
Median	107.0	113.0	187.50	98.50	Z = 3.902	P_2 < 0.001*
CRP (mg/dl)						
Min–Max	3.40–90.0	1.90–43.50	2.10–4.70	1.40–4.10	Z = 5.235	P < 0.001*
Mean ± SD	32.57 ± 21.41	14.19 ± 10.65	3.32 ± 0.89	3.01 ± 0.87	Z = 3.803	P_1 < 0.001*
Median	29.05	13.60	3.05	3.15	Z = 0.883	P_2 = 0.377
e-GFR (ml/min)						
Min–Max	4.0–11.0	14.0–29.0	43.0–119.0	100.0–128.0	Z = 5.493	P < 0.001*
Mean ± SD	7.25 ± 2.27	20.76 ± 4.31	64.75 ± 26.57	115.44 ± 9.19	Z = 4.907	P_1 < 0.001*
Median	6.0	20.0	49.50	118.0	Z = 3.841	P_2 < 0.001*

t: Student t-test; Z: Mann Whitney's test.
*Statistically significant at $P \leqslant 0.05$.
T.Cholesterol = Total cholesterol; LDL-c = Low density lipoprotein cholesterol.
HDL-c = High density lipoprotein cholesterol; TG = Triglycerides.
CRP = C-reactive protein; e-GFR = estimated glomerular filtration rate.
Group I = Patients on HD; Group II = Patients on conservative treatment.
Group III = Patients after transplantation; Group IV = Control group.
P = Comparison between control and group I; P_1 = Comparison between control and group II.
P_2 = Comparison between control and group III.

As regards the results of genotyping of fetuin-A gene polymorphisms, there was no statistically significant difference between patients of three groups and the control group or between patients as a whole and control according to the frequencies of the three fetuin-A genotype polymorphisms (C → G) (Tables 7 and 8).

Serum fetuin-A was significantly negatively correlated with IMT, BMD at spine and Z-score and positively correlated with HDL-c level but no significant correlation was found between fetuin-A and the duration of HD or transplantation. No significant correlation was found between fetuin-A and serum Ca, PO4, ALk, iPTH and e-GFR.

The negative correlation between fetuin-A and CRP and also between it and the T-score of BMD did not reach a significant value.

There was a statistical significant association between fetuin-A gene polymorphisms and the serum fetuin-A level as subjects with CG and GG genotypes had lower fetuin-A levels than those with the CC genotype (Table 9 and Fig. 3). However, no significant difference was found between fetuin-A genotypes as regards serum Ca, Po4, Alk, iPTH, CRP, IMT and BMD.

e-GFR was significantly negatively correlated with PO4 (r = −0.372, P = 0.004*); Ca×PO4 (r = −0.303, P = 0.022*); CRP (r = −0.727, P < 0.001*) and Alk (r = −0.622, P < 0.001*).

CRP was significantly positively correlated with PO4 (r = 0.314, P = 0.018*); Alk (r = 0.521, P < 0.001*) and iPTH (r = 0.442, P = 0.001*) and a significant negative correlation with albumin (r = −0.275, P = 0.038*) was seen also CRP had a significant negative correlation with BMD at the arm and pelvis (r = −0.347, P = 0.008*; r = −0.424, P = 0.001* respectively). IMT showed a significant positive correlation with PO4 (r = 0.289, P = 0.029*).

VC was significantly associated with older age, male gender, longer HD duration, lower albumin, higher LDL-c, higher carotid plaques and lower BMD at the lumbar spine and the T-score value but had no significant association with the following parameters: duration of transplantation, blood pressure, total cholesterol, TG, Ca, PO4, Ca×PO4, iPTH, CRP, fetuin A, e-GFR and IMT. Also no significant association was seen between fetuin-A gene polymorphism and VC. Patients with VC had higher CRP than those without but did not reach a significant value. (Table 10).

Table 4 Serum Ca, PO4, Ca×PO4, iPTH, alkaline phosphatase and fetuin-A in patient and controls groups.

	Group I (n = 28)	Group II (n = 17)	Group III (n = 12)	Group IV (n = 16)	Test of sig.	P value
S.Ca (mg/dl)						
Min–Max	5.80–13.10	6.70–9.60	8.10–10.20	8.70–10.30	t = 3.681	P = 0.001*
Mean ± SD	8.56 ± 1.41	8.01 ± 0.97	8.78 ± 0.59	9.63 ± 0.44	t = 6.235	P_1 < 0.001*
Median	8.55	8.10	8.70	9.65	t = 4.407	P_2 < 0.001*
S.PO4(mg/dl)						
Min–Max	3.50–9.40	3.20–7.10	3.70–4.90	2.50–4.0	t = 7.376	P < 0.001*
Mean ± S.D	5.90 ± 1.71	5.48 ± 1.13	4.41 ± 0.45	3.36 ± 0.49	t = 4.663	P_1 < 0.001*
Median	5.80	5.60	4.50	3.40	t = 4.372	P_2 < 0.001*
Ca×PO4						
Min–Max	27.01–77.29	26.24–64.32	30.34–43.12	24.0–40.40	t = 5.834	P < 0.001*
Mean ± S.D	49.99 ± 14.51	42.96 ± 12.63	38.61 ± 3.88	32.33 ± 5.13	t = 3.203	P_1 = 0.004*
Median	50.58	39.76	39.81	33.30	t = 3.541	P_2 = 0.002*
iPTH (pg/ml)						
Min–Max	52.20–3300.0	23.0–536.0	38.50–190.0	16.0–42.0	Z = 5.466	P < 0.001*
Mean ± S.D	561.69 ± 632.17	239.64 ± 146.92	92.28 ± 47.33	28.81 ± 8.31	Z = 4.505	P_1 < 0.001*
Median	461.50	214.0	76.15	29.0	Z = 4.319	P_2 < 0.001*
Alk (U/L)						
Min–Max	98.0–333.0	81.0–213.0	67.0–128.0	39.0–86.0	Z = 5.467	P < 0.001*
Mean ± SD	174.25 ± 62.24	119.76 ± 36.55	97.83 ± 19.78	61.44 ± 12.12	Z = 4.828	P_1 < 0.001*
Median	198.0	106.0	101.50	58.50	Z = 3.878	P_2 < 0.001*
Fetuin-A (g/l)						
Min–Max	0.01–0.64	0.11–0.23	0.23–0.32	0.24–0.46	Z = 2.184	P = 0.029*
Mean ± S.D	0.26 ± 0.17	0.17 ± 0.04	0.28 ± 0.03	0.35 ± 0.06	Z = 4.900	P_1 < 0.001*
Median	0.26	0.18	0.28	0.35	Z = 3.065	P_2 = 0.002*

t: Student t-test; Z: Mann Whitney's test.
S.Ca = Serum calcium; S.PO4 = Serum Phosphorus.
Ca×Ph = Calcium by Phosphorus product; Alk = Alkaline phosphatase.
iPTH = intact parathyroid hormone.
Group I = Patients on HD; Group II = Patients on conservative treatment.
Group III = Patients after transplantation; Group IV = Control group.
P = Comparison between control and group I; P_1 = Comparison between control and group II.
P_2 = Comparison between control and group III.
* Statistically significant at $P \leqslant 0.05$.

6. Discussion

CKD–MBD describes a triad of inter-related biochemical, bone and vascular abnormalities that have been linked with increased cardiovascular-related morbidity and mortality in patients with CKD and has been suggested as a non traditional risk factor for CVD in these patients [55,56].

The VC is strong predictor of CVD and all cause mortality not only in HD and peritoneal dialysis patients but also in RTRs [35,57,58].

The inhibitory role of fetuin-A on VC may include the inhibition of calcium phosphate precipitation in the serum, binding of the bone derived hydroxyapatite and limitation of dedifferentiation and apoptosis of the VSMCs [19,27,59].

There was an association between bone fragility and VC and this association was under-estimated because osteoporosis and VC were considered non-modifiable disorders of ageing [60]. It has been demonstrated that VC scores are associated with low bone turnover and low bone volume in ESRD patients [11,61].

In the present work, we studied the association between fetuin-A and its gene and both VC and BMD in patients with CKD on conservative treatment, on maintenance HD and those underwent renal transplantation.

Serum fetuin-A level was significantly lower in all patient groups than healthy controls. Other authors found similar results in ESRD patients before starting HD [30,32]. Also El-Shehaby et al. revealed that lower serum fetuin-A in HD patients was significantly associated with high calcification scores [62] and Marechal et al. showed that low serum fetuin-A level was independently associated with aortic calcifications and a higher risk of cardiovascular events and deaths in RTRs [63].

In our study, a semiquantitative assessment with plain X-rays was done to detect VC. Although this approach might be relatively crude and does not allow for differentiation between intimal and medial calcification, it has the advantages of being simple, cheap and applicable in daily clinical practice [12]. Although the use of more specific and sensitive techniques such as EBCT or spiral computed tomography might have added additional useful information, they are more sophisti-

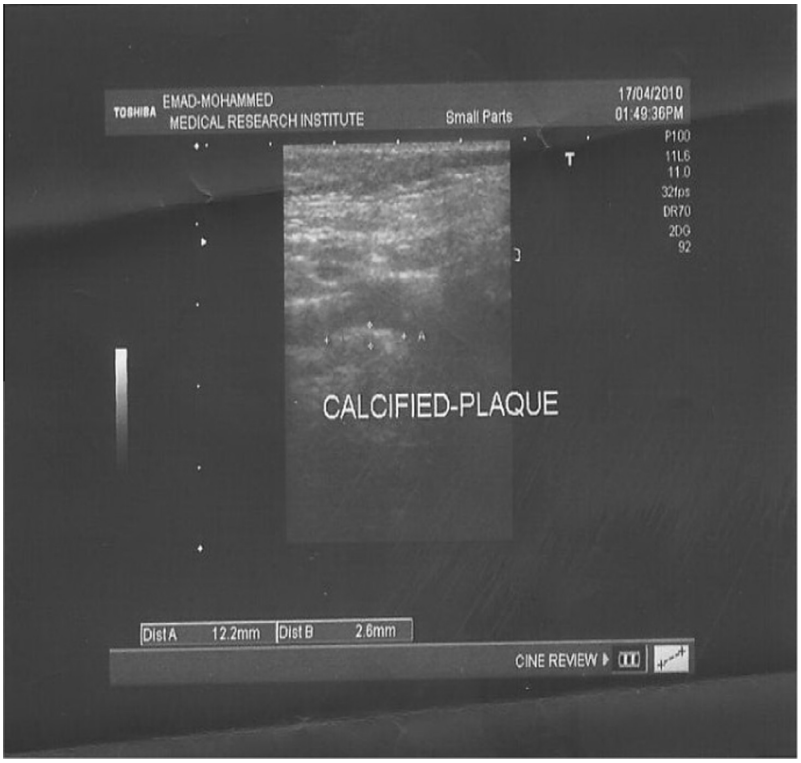

Figure 2 Carotid Doppler showing calcified plaque in haemodialyzed patient.

Table 5 Statistical analysis of radiological examination of ESRD patients and controls.

	Group I (n = 28)	Group II (n = 17)	Group III (n = 12)	Group IV (n = 16)	Test of sig.	P value
IMT (mm)						
Min–Max	0.3–1.20	0.40–0.80	0.20–0.90	0.10–0.40	Z = 5.184	$P < 0.001^*$
Mean ± S.D	0.68 ± 0.23	0.56 ± 0.13	0.60 ± 0.22	0.25 ± 0.10	Z = 4.735	$P_1 < 0.001^*$
Median	0.65	0.50	0.60	0.25	Z = 3.876	$P_2 < 0.001^*$
Plaques(+)	8 (28.6%)	4 (23.5%)	2 (16.7%)	0 (0.0)	FEp	$P = 0.036^*$ $P_1 = 0.103$ $P_2 = 0.175$
VC						
(+)	6 (21.4%)	3 (17.6%)	3 (25%)	0 (0.0)	Z = 2.830	$P = 0.005^*$
(++)	2 (7.1%)	2 (11.8%)	0 (0.0)	0 (0.0)	Z = 2.312	$P_1 = 0.021^*$
(+++)	2 (7.1%)	0 (0.0)	0 (0.0)	0 (0.0)	Z = 2.078	$P_2 = 0.038^*$
(++++)	1 (3.6%)	0 (0.0)	0 (0.0)	0 (0.0)		

FE: Fisher's exact test; Z: Mann Whitney's test.
IMT = Intima media thickness; VC = Vascular calcification.
Group I = Patients on HD; Group II = Patients on conservative treatment.
Group III = Patients after transplantation; Group IV = Control group.
P = Comparison between control and group I; P_1 = Comparison between control and group II.
P_2 = Comparison between control and group III.
* Statistically significant at $P \leqslant 0.05$.

cated, are highly time consuming and require experienced personnel [36,64].

In the present study, VC was detected in 39.3% of HD patients, 29.4% of patients on conservative treatment and 25% of those who underwent transplantation. Although other studies showed higher prevalence [6,65,66], this may be due to the reduced sample of studied patients or the use of plain X-ray for detection of VC and the use of more sensitive methods may provide more detailed information about the extension of VC in the arterial bed [64].

Blacher et al. demonstrated that 66% of HD patients had VC in the common carotid artery, abdominal aorta or in the iliofemoral vessels [6]. Higher percentage was reported by Goodman et al. in young adults on HD treatment (90% of pa-

Table 6 Statistical analysis of bone mineral density results in patient and control groups.

	Group I (n = 28)	Group II (n = 17)	Group III (n = 12)	Group IV (n = 16)	Test of sig.	P value
Arms						
Min–Max	−2.50–0.87	−0.60–0.97	0.71–1.02	−1.80–2.20	Z = 1.771	P = 0.077
Mean ± S.D	−0.65 ± 1.27	0.70 ± 0.40	0.83 ± 0.10	0.19 ± 1.07	Z = 1.893	P_1 = 0.058
Median	−0.60	0.83	0.84	0.10	Z = 2.278	P_2 = 0.023*
Pelvis						
Min–Max	−3.50–1.10	−0.80–1.21	0.95–1.22	−2.10–2.90	Z = 1.978	P = 0.048*
Mean ± S.D	−0.65 ± 1.51	0.84 ± 0.52	1.06 ± 0.08	0.54 ± 1.31	Z = 1.622	P_1 = 0.105
Median	−0.50	0.99	1.05	0.30	Z = 2.230	P_2 = 0.026*
Spine						
Min–Max	0.79–1.39	0.79–1.39	0.95–1.24	0.93–1.52	t = 2.515	P = 0.0016*
Mean ± S.D	1.0 ± 0.17	1.07 ± 0.16	1.06 ± 0.09	1.13 ± 0.17	t = 1.038	P_1 = 0.307
Median	0.99	1.04	1.03	1.07	t = 1.548	P_2 = 0.135
T-Score						
Min–Max	−3.50–1.19	−2.70–1.40	−1.10–1.10	0.82–1.50	Z = 3.748	P < 0.001*
Mean ± S.D	−0.24 ± 1.74	−0.05 ± 1.25	−0.31 ± 0.68	1.15 ± 0.18	Z = 2.651	P_1 = 0.008*
Median	0.87	0.20	−0.40	1.09	Z = 4.068	P_2 < 0.001*
Z-Score						
Min–Max	−2.5–1.40	−2.30–1.50	−2.40–1.20	0.64–1.27	Z = 4.037	P < 0.001*
Mean ± S.D	0.11 ± 1.37	0.09 ± 1.22	−0.78 ± 1.03	0.89 ± 0.14	Z = 1.299	P_1 = 0.194
Median	0.65	0.76	−0.75	0.89	Z = 3.762	P_2 < 0.001*

t: Student's t-test; Z: Mann Whitney's test.
Group I = Patients on HD; Group II = Patients on conservative treatment.
Group III = Patients after transplantation; Group IV = Control group.
P = Comparison between control and group I; P_1 = Comparison between control and group II.
P_2 = Comparison between control and group III.
* Statistically significant at $P \leqslant 0.05$.

Table 7 Distribution of fetuin-A gene between the groups.

Group	CC	CG	GG	Test of sig.
Group I	17 (60.7%)	10 (35.7%)	1 (3.6%)	χ^2 = 0.031 P_1 = 0.860
Group II	10 (58.8%)	6 (35.3%)	1 (5.9%)	χ^2 = 0.167 P_2 = 0.683
Group III	7 (58.4%)	4 (33.3%)	1 (8.3%)	FEP_3 = 0.751
Group IV	12 (75%)	3 (18.8%)	1 (6.2%)	χ^2 = 1.263 P_4 = 0.261
MCp	0.893			

χ^2: Chi square test; MC: Monte Carlo test.
Group I = Patients on HD; Group II = Patients on conservative treatment.
Group III = Patients after transplantation; Group IV = Control group.
P_1 = Comparison between CC with each of CG and GG.
P_2 = Comparison between CG and GG.

Table 8 Comparison between patients and control group according to the frequencies of fetuin-A genotype polymorphisms (C → G).

Fetuin-A genotypes	Patients	Control	χ^2 (P)
CC	34 (59.6%)	12 (75%)	1.263 (0.261)
CG	20 (35.1%)	3 (18.7%)	
GG	3 (5.3%)	1 (6.3%)	

χ^2: Chi square test between genotype CC and other genotypes between patients and control.

tients aged 20–30 years) [65]. Goldsmith et al. found that the prevalence of VC increased from 39% at the start of dialysis to 92% after 16 years of HD [66], in another study Hernandez et al. reported that 24.4% of renal transplant candidates had VC on a plain abdominal or pelvic X-ray before renal transplantation [36]. Although successful RT may improve some risk factors associated with the uremic state, VC may extend or at least do not regress in a considerable proportion of RTRs and remain at an increased risk for CVD compared to the general population [36].

In our study, BMD showed lower values in all patient groups than the control which is similar to other results [67,68]. Although low BMD is a common finding in dialysis patients, Taal et al. demonstrated that dialysis patients with bone loss have higher rates of cardiovascular death compared with patients with normal BMD [69].

Table 9 Correlation of serum fetuin-A with different parameters.

	R	*P*
Duration of HD (months)	−0.010	0.961
Duration of transplantation (months)	−0.207	0.519
Serum Ca (mg/dl)	−0.246	0.065
Serum phosphorus (mg/dl)	−0.026	0.849
iPTH (pg/ml)	−0.034	0.800
Albumin (g/dl)	−0.186	0.167
Alk (U/l)	−0.100	0.461
LDL-c (mg/dl)	−0.003	0.982
HDL-c (mg/dl)	0.319	0.016*
e-GFR (ml/min)	0.072	0.592
CRP (mg/dl)	−0.096	0.475
IMT (mm)	−0.296	0.025*
Arm (BMD)	−0.214	0.109
Pelvis (BMD)	−0.103	0.445
Spine (BMD)	−0.355	0.007*
T-score (BMD)	−0.180	0.181
Z-score (BMD)	−0.277	0.037*
Fetuin genotype (CC/CG/GG)	−0.246	0.036*
Fetuin genotype (CC/CG + GG)	−0.244	0.038*

r_s: Spearman coefficient.
HD = Hemodialysis.
Alk = Alkaline phosphatase; iPTH = Intact parathyroid hormone.
LDL-c = Low density lipoprotein cholesterol.
HDL-c = High density lipoprotein cholesterol.
e-GFR = estimated glomerular filteration rate; CRP = C. reactive protein; IMT = Intima media thickness; BMD = Bone mineral density.
* Statistically significant at $P \leqslant 0.05$.

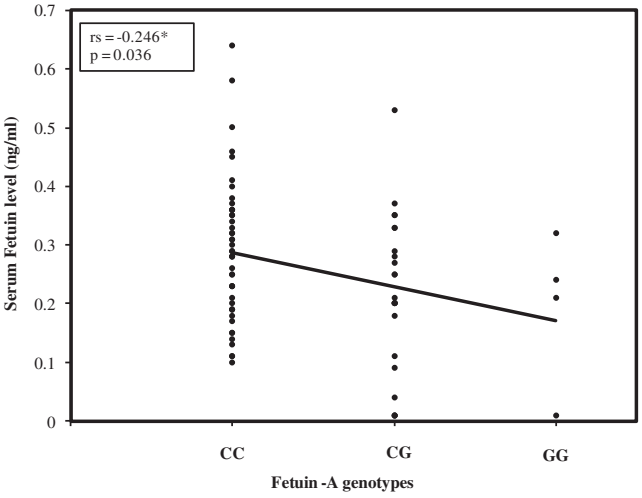

Figure 3 Univariate correlation between serum fetuin-A level and fetuin-A genotypes in the studied subjects.

IMT was significantly higher in all patient groups than the control group with demonstration of plaques in 28.6% of group I, 23.5% of group II and 16.7% of group III. These results were similar to other studies in CKD and HD patients [70,71]. Nafar et al. found that IMT increased over a short time in RTRs [72].

Serum fetuin-A level did not show significant correlation with e-GFR, Ca, PO4, Ca×PO4, iPTH, Alk, CRP, albumin, T-score of BMD or VC. It showed only a significant negative correlation with IMT.

Stenvinkel et al. found significant correlation between the fetuin-A level and both albumin and CRP and no significant correlation between the fetuin-A level and e-GFR [30]. Also Ketteler et al. reported an inverse relationship between the serum fetuin-A level and CRP [33]. Hamano et al. did not find a significant correlation between the serum fetuin-A level and CACS but found a significant positive correlation between the fetuin-A level and serum albumin [73]. Ford et al. demonstrated that the fetuin-A level was correlated with PO4 but not with CRP [20]. Moe et al. also found no correlation between the serum fetuin-A level and VC, serum levels of Ca, PO4 or iPTH [13].

Jung et al. [74], Hermans et al. [75]and Ix et al. [76] reported that low serum fetuin A is not an independent predictor of VC in patients with CKD which was similar to our study.

The absence of significant negative correlation between fetuin-A and CRP can be explained by the small number of patients studied and the large proportion of patients in whom CRP was not elevated and we did not use the high sensitive method for the detection of CRP. Our results were similar to those observed by Schaalan et al. who demonstrated negative correlation between CRP and fetuin-A that did not reach a significant value in HD children [77].

We observed that patients with VC had higher CRP levels but with no statistically significance difference compared with patients without calcification. Other studies showed similar results [10,78,79]. DeLoach et al. found that CRP was associated with the presence of aortic calcification at the time of transplant and aortic calcification progression post transplant [5]. Turkmen et al. found no difference in the CRP level between patients according to CACS in HD or PD patients [80]. It was suggested that CRP may be one of the key mediators of vessel wall calcification in HD patients [81]. Inflammation is an integral component of atherosclerosis that may worsen in severity by reducing fetuin-A synthesis and other circulating calcification inhibiting factors [33] and the present work confirmed the association between higher CRP serum levels and severe VC and also the negative significant correlation between fetuin-A and IMT as fetuin-A is regulated as a negative acute phase protein and low fetuin-A and high CRP may represent the same biological event [35].

Patients with VC were significantly older, predominantly male with a higher duration of HD, higher LDL-c, lower albumin level and lower BMD at the spine and lower T-score. These results are in accordance with previous studies as Coll et al. who demonstrated that patients with VC were significantly older, predominantly male but showed no differences in time of dialysis, BMI, blood pressure and lipid profile than those without VC [78]. McCullough et al. concluded that CAC was strongly correlated with older age, male gender and longer time of dialysis [82].

Asci et al. also found that increasing CACs were associated with older age, male gender, longer HD vintage and lower albumin and that there were no statistically significant differences between CACs and Ca, PO4, iPTH, triglycerides, total

Table 10 Demographic and biochemical characteristics of the study population according to vascular calcification.

	Vascular calcification (n = 19)	No vascular calcification (n = 38)	Test of sig.	P value
Male sex	11 (57.9%)	12 (31.6%)	χ^2 = 3.645	P = 0.056
Age (years)	52.26 ± 9.77	45.37 ± 11.95	t = 2.175	P = 0.034*
BMI (kg/m^2)	25.96 ± 4.94	25.69 ± 5.84	t = 0.176	P = 0.861
Hypertension	10 (52.6%)	18 (47.4%)	χ^2 = 0.140	P = 0.708
IHD	4 (21.1%)	10 (26.3%)	FEp	P = 0.754
Duration of HD (months)	105.0 ± 64.43	36.59 ± 19.94	Z = 3.296	P = 0.001*
Duration of transplantation(months)	31.0 ± 10.58	31.22 ± 11.98	Z = 0.277	P = 0.782
MBP (mmHg)	102.25 ± 8.26	102.86 ± 12.0	t = 0.233	P = 0.825
SBP (mmHg)	132.63 ± 12.95	135.79 ± 17.42	t = 0.698	P = 0.488
FBS (mg/dl)	95.58 ± 10.35	94.53 ± 9.65	t = 0.379	P = 0.706
Urea (mg/dl)	123.37 ± 29.84	108.76 ± 43.31	t = 1.319	P = 0.193
Creatinine (mg/dl)	6.78 ± 3.41	5.63 ± 3.74	Z = 1.355	P = 0.175
Albumin (g/dl)	3.07 ± 0.68	3.53 ± 0.56	t = 2.722	P = 0.009*
T.cholesterol (mg/dl)	154.68 ± 35.23	117.29 ± 37.55	Z = 1.837	P = 0.066
LDL-c (mg/dl)	118.26 ± 32.73	94.68 ± 27.13	t = 2.706	P = 0.009*
HDL-c (mg/dl)	37.61 ± 12.36	40.89 ± 16.10	Z = 0.542	P = 0.588
TG (mg/dl)	138.76 ± 59.17	119.42 ± 49.15	Z = 1.117	P = 0.264
S.ca (mg/dl)	8.16 ± 0.98	8.58 ± 1.25	t = 1.281	P = 0.205
S.PO4 (mg/dl)	5.51 ± 1.52	4.82 ± 1.57	t = 1.642	P = 0.105
Ca×PO4	44.90 ± 13.42	41.80 ± 12.90	t = 0.891	P = 0.376
ALk (U/L)	150.47 ± 65.67	137.63 ± 54.89	Z = 0.847	P = 0.397
e-GFR (ml/min)	20.79 ± 27.52	24.68 ± 24.56	Z = 1.179	P = 0.238
CRP	28.40 ± 25.30	17.20 ± 15.92	Z = 1.727	P = 0.086
iPTH	372.71 ± 726.04	363.87 ± 325.23	Z = 0.821	P = 0.412
Fetuin A	0.27 ± 0.15	0.22 ± 0.12	Z = 0.973	P = 0.330
Fetuin-A genotype			MCp	P = 0.327
CC	22 (57.9%)	12 (63.2%)		
CG	15 (39.5%)	5 (26.3%)		
GG	1 (2.6%)	2 (10.5%)		
IMT (mm)	0.67 ± 0.19	0.61 ± 0.21	Z = 1.192	P = 0.233
Plaques (+)	8 (42.1%)	6 (15.8%)	χ^2 = 4.734	P = 0.03*
ARM	0.60 ± 0.61	−0.20 ± 1.27	Z = 0.254	P = 0.799
Pelvis	0.78 ± 0.74	−0.16 ± 1.48	Z = 1.407	P = 0.160
Spine	0.96 ± 0.13	1.07 ± 0.15	t = 2.837	P = 0.006*
T-score	−1.64 ± 1.29	0.53 ± 0.78	Z = 5.289	P < 0.001*
Z-score	−1.28 ± 1.36	0.35 ± 0.82	Z = 4.056	P < 0.001*

χ^2: Chi square test; MC: Monte Carlo test; FE: Fisher's Exact test.
Z: Mann Whitney's test; t: Student's t-test.
BMI = Body mass index; IHD = Ischemic heart disease.
HD = Hemodialysis; MBP = Mean blood pressure; SBP = Systolic blood pressure; FBG = Fasting blood glucose.
T.Cholesterol = Total Cholesterol; LDL-c = Low density lipoprotein cholesterol.
HDL-c = High density lipoprotein cholesterol; TG = Triglycerides.
Ca×Po4 = Calcium by phosphorus product; ALK = Alkaline phosphatase.
e-GFR = estimated glomerular filteration rate; CRP = C-reactive protein.
iPTH = intact parathyroid hormone; IMT = Intima media thickness.
* Statistically significant at $P \leqslant 0.05$.

cholesterol and CRP [83]. Moe et al. demonstrated that the duration of dialysis and age were the only factors that were correlated with coronary calcification and thus, it is tempting to speculate that decreasing fetuin-A level during renal replacement therapy may be one reason for accelerated VC [39].

In the present study, no significant correlation was found between VC and time of transplantation which may be explained by the small number of transplanted patients. Seyahi et al. found that longer time of transplantation was associated with CAC [2]. It was not clear whether the progression of CAC slows down or reverses following renal transplantation. However the authors concluded that renal transplantation slows down but does not halt CAC progression [2].

We found that patients with VC had significant lower albumin levels than those without. It was demonstrated that advancement of VC in ESRD patients was associated with inflammatory markers. Wang et al. confirmed the link between malnutrition, markers of inflammation, atherosclerotic vascular and cardiac calcification in PD patients and considered changing the term to "malnutrition-inflammation-atherosclerosis-calcification syndrome"[35]. Although it was proposed that disorders of mineral metabolism may play a role in the genesis of VC in ESRD patients [73]. We observed no significant difference between patients with VC and those without as regards Ca, Po4, Ca×Po4 product, Alk, iPTH and the serum fetuin-A level although those with VC had lower Ca, higher

PO4 and higher iPTH but did not reach statistically significant results possibly due to reduced sample size. Barreto et al. found no association between CACs and Po4, Ca, iPTH or Alk [79].

Braun et al. failed to find any relationship between various parameters of calcium-phosphate balance, but they noticed that the calcium score correlated with BMD in the lumbar spine [84].

These findings suggest that bone metabolism and turnover may affect the degree of mineral content deposition within the vascular wall and this points to a possible relationship between VC and bone turnover [34].

Rodriguez-Garcia et al. showed that only age and time on RT were associated with an increased risk of VC and the prevalence of vertebral fractures and serum Ca, PO4, Ca×PO4 and iPTH were not significantly associated with the prevalence of any type of VC [43]. Also Seyahi et al. found similar results in RTRs [2].

Experimental data have clearly shown that VSMCs can modify their phenotype becoming osteoblast like cells able to induce VC .This fact has opened a fascinating area of research between vascular and mineral metabolism [43].

The association between osteoporosis and VC was not definitively explained and there is uncertainty whether the two disease processes are truly linked. VC in and by itself could affect bone metabolism; arterial stenosis could decrease peripheral blood supply, suppressing bone cell function, or atherosclerotic disease might limit physical activity, leading to bone loss [85].

Inflammation may promote VC and osteoporosis has an important inflammatory component, this might be part of the pathway linking VC and bone loss [60].

It was demonstrated that patients with the highest degree of aortic calcification had the lowest bone density and those with the most severe aortic calcification had not only a lower bone mass but also a higher incidence of new osteoporotic fractures [61]. Rodriguez-Garcia et al. demonstrated that VC in medium caliber arteries was associated with an increased risk of vertebral fractures but there was no relationship between VC and peripheral osteoporotic fractures [43]. Asci et al. found a positive correlation between bone turnover and CAC [83].

In our study we demonstrated that patients with VC had lower BMD than those without which was evident in the spine and total T-score. Filgueira et al. concluded that low vertebral bone density (VBD) was associated with the presence of CAC in nondialyzed CKD patients and they suggested that low VBD might constitute another nontraditional risk factor for CVD in CKD patients considering that bone loss and CVD share similar etiopathogenic mechanisms [86].

In an attempt to know more about the pathogenesis of increased risk of VC and atherosclerosis in patients with CKD, restriction enzyme analysis was done for the detection of restriction fragment length of fetuin-A; (C → G);Thr256Ser gene polymorphisms.

There was no statistically significant difference between CKD patients and the control group according to the frequencies of the three fetuin-A genotype polymorphisms (C → G) but there was a statistically significant association between fetuin-A gene polymorphisms and the serum fetuin-A level as subjects with CG and GG genotypes had lower fetuin-A level than those with CC genotype.

Stenvinkel et al. demonstrated that Swedish dialysis patients with fetuin-A 256Ser/Ser (allele G) had lower serum fetuin-A levels and were associated with higher cardiovascular mortality rate [30]. Verduijn et al. studied HD patients who were genotyped for the Thr256Ser polymorphism and found that carriers of a serine allele displayed lower fetuin-A levels. A small increased mortality risk was observed for the Thr/Ser and Ser/Ser genotype compared with the Thr/Thr [87]. Also Axelsson et al. found that fetuin-A (C → G); Thr256Ser gene polymorphisms influenced circulating levels of fetuin-A [88].

However Cozzolino et al. found that in both Italian HD patients and the control group, the distribution of fetuin-A gene did not show significant association between low serum fetuin-A and fetuin-A (C → G); Thr256Ser gene polymorphism [89]. Also

Zeidan et al. demonstrated similar results [32].

Probably, the reason that some recent clinical results are different from our findings may be explained by the differences in races and other fetuin-A single nucleotide polymorphisms not yet known may affect its serum level.

With fetuin-A being a negative acute phase glycoprotein, it is plausible that fetuin-A links to VC by reflecting the risk of inflammation. Persistent inflammation in dialysis patients may serve as a catalyst and in the toxic uraemic milieu, modulates the effects of other concurrent risk factors, which would thereby exacerbate the VC process [30,79]. As osteoporosis has an important inflammatory component, the latter might be part of the pathway linking VC and bone loss [60].

However beyond the effect of inflammation, the role of serum fetuin-A level in VC may be far more complex than previously thought [80].

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