

Phosphorylated fetuin-A-containing calciprotein particles are associated with aortic stiffness and a procalcific milieu in patients with pre-dialysis CKD

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Abstract

Background. Vascular stiffening occurs in normal ageing and is accelerated in chronic kidney disease (CKD). Vascular calcification contributes to this stiffening and to the high incidence of vascular morbidity and mortality in this population. A network of inhibitors work in concert to reduce mineralization risk in extra-osseous tissue. Fetuin-A is an important systemic inhibitor of ectopic calcification. A fraction of the total circulating fetuin-A interacts with mineral ions to form stable colloidal complexes, calciprotein particles (CPP), preventing deposition. We sought to assess whether CPP fetuin-A levels were associated with procalcific factors and aortic stiffness in a cohort of patients with Stages 3 and 4 CKD.

Methods. We measured fetuin-A CPP levels, serum inflammatory markers [C-reactive protein (CRP), interleukin-6, tumour necrosis factor- α], oxidized low-density lipoprotein (oxLDL), bone morphogenetic protein-2 (BMP-2) and -7 (BMP-7) and aortic pulse wave velocity (APWV) in a cohort of 200 CKD patients. Serum measurements were also made in 78 healthy controls. CPP fetuin-A phosphorylation was characterized by phosphate-affinity gel chromatography.

Results. Fetuin-A-containing CPPs were only detectable in the serum of CKD patients. Inflammatory markers, oxLDL and BMP-2 levels were all significantly higher in the CKD than control subjects. CPP fetuin-A levels were independently associated with serum phosphate, high-sensitivity C-reactive protein, oxLDL, BMP-2/7 ratio and inversely with estimated glomerular filtration rate (model $R^2 = 0.51$). After adjusting for confounders, CPP fetuin-A levels were independently associated with APWV. Only phosphorylated fetuin-A was present in serum CPP.

Conclusion. Increased CPP fetuin-A levels reflect an increasingly procalcific milieu and are associated with increased aortic stiffness in patients with pre-dialysis CKD.

Keywords: aortic stiffness; bone morphogenetic proteins; fetuin-A; inflammation; vascular calcification

Introduction

Vascular calcification is prevalent in patients with chronic kidney disease (CKD) and is a significant risk factor for cardiovascular (CV) mortality [1, 2]. In CKD, a combination of mineral imbalance, chronic inflammation and loss of local and systemic inhibitors give rise to an extracellular environment that can trigger mineralization within the vasculature. Increased medial calcification of the central vessels leads to a loss of compliance of the arterial wall [3], and this appears to be a major contributor to vascular stiffening [4, 5]. Arterial stiffening can be measured by aortic pulse wave velocity (APWV), and this is independently associated with the rate of decline in renal function in patients with CKD [6] as well as being a strong independent predictor of mortality in dialysis patients [7].

Knockout animal models have demonstrated the importance of a network of inhibitors in controlling calcification [8]. Fetuin-A is an abundant liver-derived plasma protein that has multiple roles in regulation of mineralization [9, 10]. Fetuin-A-deficient mice develop severe CV and soft tissue calcification [11, 12] and may serve as a model for patients with CKD where lower serum fetuin-A concentrations have been associated with increases in calcification scores, arterial stiffness and mortality [13–15]. We have previously shown that lower plasma fetuin-A concentrations are associated with increased aortic stiffness >12 months in patients with mild-to-moderate CKD [16].

Fetuin-A has a high affinity for mineralized bone matrix [17] and forms colloidal complexes with calcium apatite in serum [calciprotein particles (CPP)] and other supersaturated mineral solutions preventing crystal growth and aggregation

[18, 19]. It has been postulated that these CPPs serve as mineral chaperones, sequestering mineral that may otherwise seed mineralization at ectopic sites and facilitating transport and clearance from the body fluids [20]. Recent work by Hamano *et al.* [21] suggests that estimates of this specific fraction of total circulating fetuin may provide a better measure of extraskeletal mineral stress in patients with CKD.

There is also accumulating evidence that chronic inflammation and increased oxidative stress, commonly seen in patients with CKD [22], may act as major drivers towards aberrant calcification [23]. Indeed, it has been suggested that mineral crystals may themselves be pro-inflammatory, creating a vicious cycle of inflammation and calcification [24]. *In vitro* studies have shown that dysregulated mineral metabolism and inflammatory cytokines such as tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) can induce the transformation of vascular smooth muscle cells (VSMC) to osteochondrocytic-like phenotype. This event appears to be important in the development of vascular calcification and is associated with increased expression of bone-related regulatory proteins [e.g. alkaline phosphatase (ALP), osteocalcin and osteopontin] [23, 25, 26].

Bone morphogenetic protein-2 (BMP-2) is a potent osteogenic cytokine that plays a key role in vascular calcification pathogenesis [27] and sits at a point where inflammatory and oxidative signals converge. Early work by Boström *et al.* [28] demonstrated the expression of BMP-2 in atherosclerotic lesions and the ability of explanted aortic cells to calcify in a manner similar to bone cell cultures. BMP-2 signalling leads to up-regulation of the bone-transcription factors Runx2 and Msx and phosphate transporter, Pit-1 in VSMCs [29]. BMP-2 can also induce the generation of reactive oxygen species and VSMC apoptosis [30–32]. Oxidized low-density lipoprotein (oxLDL) has also been shown to induce the production of TNF- α from activated macrophage and increases vascular BMP-2 expression, activating Wnt signalling pathways and osteogenic gene pathways [33, 34]. In contrast, BMP-7, another member of the transforming growth factor- β (TGF- β) superfamily, has been shown to reduce vascular calcification in high-fat fed, low-density lipoprotein receptor-deficient mice [35] and in another murine model of low turnover osteodystrophy [36]. Furthermore *in vitro*, BMP-7 also maintains the contractile VSMC phenotype [37].

The aims of the present study were to assess the relationship between CPP fetuin-A levels with emerging procalcific triggers (BMP-2, oxLDL, inflammatory mediators) and aortic stiffness in a cohort of 200 patients with CKD Stages 3 and 4. Additionally, we aimed to evaluate the phosphorylation pattern of fetuin-A species present within this patient group.

Materials and methods

CPP isolation and measurement

CPP fetuin-A levels were determined indirectly as described by Hamano *et al.* with minor modification [21]. In the paper by Hamano *et al.*, CPPs were sedimented from serum after centrifugation for 2 h at 16 000 g and 4°C. These centrifugation parameters were adopted from studies on rat serum CPP [38]. CPP fetuin-A levels were then calculated by the difference in serum (assumed to be total) fetuin-A and supernatant (monomeric)

concentrations. Before applying this analytical protocol to our study, we evaluated the adequacy of centrifugation at this g to pellet all the CPPs present in serum. Figure 1A shows the sedimentation profiles of CPP in

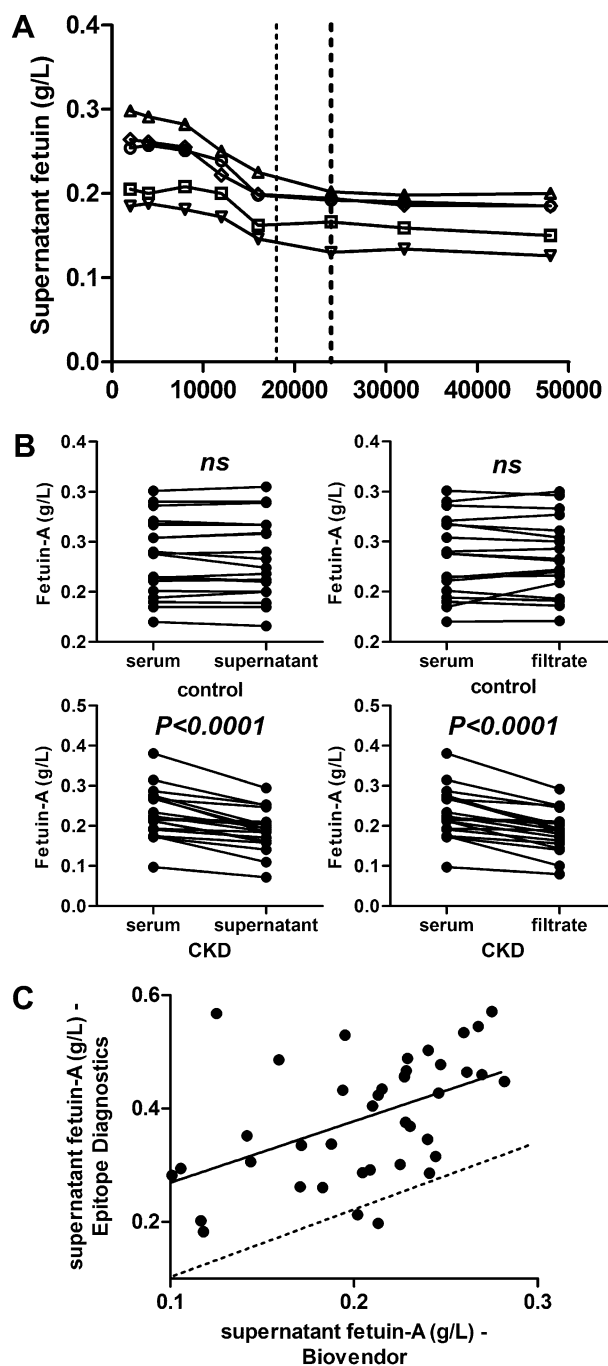


Fig. 1. Validation of CPP isolation protocol. (A) Fetuin-A sedimentation profiles in five serum samples from patients with pre-dialysis CKD showing variation in speed at which CPP pellet in different samples. First dotted line indicates centrifugation speed used by Hamano *et al.* [21], the second bolder line indicates the speed used in the current study. (B) Comparison of centrifugation (24 000 g, for 2 h at 4°C) and filtration methods of serum fractionation revealing similar reduction in serum fetuin-A after either treatment. Both methods show evidence of CPP in serum from pre-dialysis CKD patients ($n = 15$, $P < 0.001$, Wilcoxon signed-ranks test) but not in healthy control samples ($n = 15$). (C) Comparison of supernatant fetuin-A concentration measured using Biovendor and Epitope Diagnostic ELISA kits ($n = 40$) showing a lack of agreement between assays. Continuous line shows Deming fit and the dotted line shows line of equality.

five serum samples from patients with pre-dialysis CKD. This demonstrates significant between-sample differences in the speed at which all CPPs are pelleted when spun for 2 h, ranging from 16 000 to 24 000 g. This presumably reflects variation in CPP size and sample matrices. To validate this further, we compared the reduction in serum fetuin-A after centrifugation at 2 h at 24 000 g and 4°C in 15 pre-dialysis CKD and 15 control samples with that observed after fractionation using a 300-kDa molecular weight cut-off centrifugal filtration unit (Centrisart; Sigma–Aldrich, Dorset, UK) (See Figure 1B). These results showed close agreement (within 94%).

Given our previous report highlighting the poor agreement between commercial fetuin-A enzyme-linked immunosorbent assay (ELISA) kits [28], we compared supernatant fetuin-A concentration in 40 CKD patients using two different kits: Biovender (Brno, Czech Republic) and Epitope Diagnostics Inc. (San Diego, CA) (see Figure 1C). Although these measurements were strongly correlated ($r = 0.587$, $P < 0.001$), there is still evidence of substantial disagreement between assays that purportedly measure the same analyte. Deming regression: intercept = -0.174 [95% confidence interval (CI) -0.477 to 0.130 , slope = 2.701 (95% CI 1.292 to 4.111)]. Bland–Altman analysis: mean bias Epitope versus Biovender was $57.9 \pm 26.3\%$. Hamano *et al.* [21] made similar kit comparisons ($n = 13$, $R^2 = 0.440$, $P < 0.05$) but interpreted this finding differently. The use of simple correlations to assess the agreement between two methods is inappropriate [66]. We have demonstrated that the Epitope Diagnostics kit used by Hamano *et al.* to be less sensitive and precise than the Biovender assay used in the current study. Indeed, some samples in their study showed an apparent increase in fetuin-A concentration (negative reduction ratio) after centrifugation suggesting a lack of analytical precision.

In the present clinical study, serum fetuin-A was measured by ELISA following centrifugation of clotted blood samples (10 min, 2000 g). Aliquots (500 μ L) of each serum sample were then subjected to further centrifugation at 24 000 g for 2 h. The supernatant was then reanalysed for fetuin-A using the same ELISA assay. CPP fetuin-A levels were expressed as a percentage of the total serum concentration using the following formula: (serum fetuin-A – supernatant fetuin-A)/serum fetuin-A \times 100. In a small series of patients ($n = 15$), these pellets were washed with 150 μ L ice-cold 0.9% saline and then subjected to further biochemical characterization.

Biochemical characterization of fetuin-A-containing CPP

For calcium, phosphate and total protein analysis, pellets were dissolved in 35 μ L 150 mM HCl overnight at room temperature with gentle mixing. Calcium content was determined by ultrasensitive graphite furnace atomic absorption spectrophotometry as previously described [39]. Phosphate was determined by reaction with ammonium molybdate in sulphuric acid and total protein by reaction with benzethonium chloride after neutralization with 2 M NaOH. For the detection of specific proteins, pellets were dissolved in 35 μ L 20 mM Tris–HCl (pH 7.4) containing 60 mM ethylenediaminetetraacetic acid (EDTA) overnight at room temperature with gentle mixing. Albumin, immunoglobulin G, C3, C4 and fibrinogen were all measured by immunonephelometry using commercially available reagents on a Dade Behring ProSpec analyser. Fetuin-A (total), osteoprotegerin (OPG), sRANKL and BMP-2 were measured as by sandwich ELISA.

Phospho-fetuin-A isoform analysis

Rat CPPs have been shown to contain predominantly phosphorylated fetuin-A [38]. We used the same technique to characterize the phosphorylation status of fetuin-A in human CPP circulating in serum. In 20 pre-dialysis CKD patients, pelleted serum CPPs were further analysed for the presence of different fetuin-A phosphoisoforms. Pellets were washed twice in 0.9% ice-cold saline and then dissolved in 35 μ L 150 mM HCl overnight (12 h) at room temperature. Samples were neutralized with 2 M NaOH and then mixed 1:1 with Laemmli loading buffer and boiled for 5 min prior to electrophoresis. Separation of fetuin-A into its different phosphorylated states was achieved by phosphate-affinity sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a polyacrylamide-bound Mn^{2+} -phosphate-binding tag (Phos-tag, Nard Institute, Hyogo, Japan) that retards phosphorylated proteins within the gel matrix depending on the number and site of the phosphate group [40]. For dephosphorylation, neutralized samples were diluted 1:5 in 0.10 M Tris–HCl pH 9.0 containing 0.05 M $MgCl_2$, 0.1 mM dithiothreitol, 0.5 U/ μ L high activity bovine intestinal ALP (~ 6000 U/mg protein) and incubated overnight at 37°C. Reactions were stopped by addition of equal volume of

Laemmli loading buffer and samples heated to 95°C for 5 min prior to electrophoresis. Serum or dissolved pellets were subjected to SDS–PAGE at 25 mA using 10% gels containing 85 μ M Mn^{2+} -Phos-tag (NARD Institute). Gels were soaked in 3 mM EDTA and then electroblotted onto Hybond-P membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) overnight at 30 V and 4°C. Blots were detected using rabbit anti-human fetuin-A polyclonal antibody (Biovender) and the WesternDot 625 western blot kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Membranes were imaged under ultraviolet transillumination. For reproduction, the image was converted to grey scale and then inverted.

In vitro serum CPP formation

Fetuin-A was purified from the supernatant of pooled CKD patient serum after centrifugation at for 2 h at 24 000 g and 4°C. Briefly, 10 mL serum was subjected to gel filtration with a HiPrep Sephacryl 26/60 S-300HR column (GE Healthcare) equilibrated with 50 mM Tris–HCl, 150 NaCl, 5 mM EDTA (pH 7.4). Appropriate fractions were pooled and mixed with a wheat germ agglutinin resin (Thermo Scientific Pierce) to isolate glycoproteins before affinity extraction with anti-human fetuin-A antibody (Biovender). Purity was confirmed by SDS–PAGE analysis ($>98\%$). Dephosphorylated fetuin-A was generated by enzymatic dephosphorylation with 5 U/L ALP and then purified by affinity chromatography as before. Phosphorylation status was confirmed by phos-tag SDS–PAGE. Fetuin-A-depleted serum was produced by centrifugation of patient serum at for 2 h at 24 000 g and then incubation of the supernatant with anti-human fetuin-A antibody coupled to agarose resin (Thermo Scientific) for 12 h at room temperature with gentle mixing. Fetuin-A depletion was confirmed by ELISA (<0.5 μ g/L). The resultant depleted serum (500 μ L) was then reconstituted with either native or dephosphorylated fetuin-A to a final concentration of 0.70 mg/mL. CPP formation was initiated by mixing serum with 250 μ L concentrated buffered salt solution containing 10 mM $CaCl_2$, 6 mM Na_2HPO_4 in 50 mM Tris–HCl at pH 7.4 and incubating for 12 h at 24°C. Fetuin-A-containing CPPs were estimated by the reduction in supernatant fetuin-A concentration after high-speed centrifugation as previously described.

Study participants

Two hundred participants were enrolled in a prospective study of CV risk in patients with Stages 3 and 4 CKD [estimated glomerular filtration rate (eGFR) 15–59 mL/min/1.73m²]. Details of this study design and cohort characteristics have been previously published [41]. These patients were predominantly attending nephrology outpatient clinics at Brighton and Sussex University Hospitals NHS Trust from March 2006 to September 2010. A full history covering renal disease, CV disease and other risk factors was obtained at entry to the study. All participants were treated with the aim of achieving UK Renal Association targets for management of blood pressure in CKD; the choice of anti-hypertensive medication remained at the discretion of the patient's clinician but generally followed British Hypertension Society guidelines [42].

Control subjects had no history of CV disease (exclusion criteria: previous myocardial infarction, stroke, heart failure or receiving lipid-lowering/anti-hypertensive therapy), Type 2 diabetes mellitus, malignancy, recent infection or trauma, with an estimated glomerular filtration rate (eGFR > 60 mL/min/1.73m²) and a urinary total protein:creatinine ratio (<10 mg/mmol).

Patients gave written informed consent, and the study was approved by local regional ethics committee and conducted in accordance with the Declaration of Helsinki.

APWV measurements

APWV was measured using Complior™ (Colson, Les Lilas, France) as previously described [6] and has been validated elsewhere [43]. APWV measurements were available for 185 subjects, while no measurement was possible in 15 (8 had a pulse waveform which was not detected by the software, 3 had had major vascular surgery and 4 had impalpable femoral or carotid pulses).

Biochemical analysis

Clotted blood samples were collected at baseline and 12-month follow-up appointment, allowed to clot for 30 min and then centrifuged for 10 min at 3000 g. The serum was stored in aliquots at -80°C until batched analysis.

eGFR was calculated using the four-variable equation derived from the Modification of Diet in Renal Disease study [44]. Serum high-sensitivity

C-reactive protein (hs-CRP) was measured by particle-enhanced immunonephelometry on the Dade Behring ProSpec analyser (Siemens, Camberley, UK). Intra-assay and inter-assay imprecision were <3.8 and 5.2% respectively, limit of detection was 0.175 mg/L. Plasma intact parathyroid hormone (PTH) and serum β -isomerized C-terminal telopeptides (β -CTX) were measured using Elecsys reagents for the Modular Analytics E170 immunoanalyser (Roche Diagnostics, Burgess Hill, UK).

Serum fetuin-A, oxLDL, IL-6, TNF- α , BMP-2 and BMP-7 were all measured using commercially available ELISA kits. The source and performance characteristics of these assays are given as Supplementary material.

Statistical analysis

APWV was adjusted for mean arterial pressure as previously described [6].

Skewed data were log transformed. For trend analysis across tertiles of CPP fetuin-A, we used analysis of variance with Bonferroni correction (or Kruskal–Wallis test with Dunn's post-test) for continuous variables and χ^2 tests (or Fisher exact equivalent) for categorical variables (P-values are for trend). For hypothesis testing, sequential multivariate models were constructed to assess the relationship between CPP fetuin-A levels and APWV. To aid interpretation of log-transformed variables, unstandardized *B*-values are given throughout. Covariates were defined a priori, based on previously described associations with either APWV (outcome) or CPP fetuin-A (primary predictor) or if found to be significant in univariate analysis. For exploratory modelling, all variables that had a P-value of <0.1 in univariate analysis were entered into the model.

For serial data, the change in each variable was expressed as a percentage relating to the baseline value. P-values <0.05 from two-sided tests were considered significant unless otherwise stated. Analyses were performed using SPSS for Windows version 19.0 (IBM, Portsmouth, UK).

Results

Biochemical characterization of CPP in CKD serum

Consistent with previous reports [21], fetuin-A-containing CPPs [or fetuin mineral complex (FMC)] were undetectable after centrifugation of control serum at 24 000 *g*. CPP isolated from CKD serum were analysed for protein and mineral content. By protein mass, fetuin-A was found to be the most abundant protein (65%), along with albumin (26%) and fibrinogen (3%) and trace amounts of OPG, sRANKL and BMP-2 (each <1%). Other major serum proteins, IgG, C3 and C4 were not detectable within the lower limits of these assays, suggesting minimal contamination from ordinary serum components (see Figure 2A). In terms of mineral content, CPP contained a mean 0.50 ± 0.16 $\mu\text{g/mL}$ calcium and 0.38 ± 0.23 $\mu\text{g/mL}$ phosphate. Sedimented calcium was significantly correlated with CPP fetuin-A levels and inversely associated with eGFR. Plasma-adjusted calcium concentration, on the other hand, was not significantly related to either pellet Ca or eGFR (see Figure 2B–E).

Fetuin-A phosphorylation

It has recently been demonstrated that only fully phosphorylated fetuin-A is present in the CPPs from the serum of a rat model with adenine-induced renal failure [38]. Serum fetuin-A was found to be predominantly unphosphorylated (mean $88.7 \pm 6.3\%$) but was also present in two discrete phosphorylated states (Figure 3A, Lanes 1–2). CPP-containing fetuin-A, on the other hand, showed almost exclusively phosphorylated isoforms (Figure 3A, Lanes 3–8). The finding of only phosphorylated fetuin-A within CPP is unexpected as fetuin-A phosphorylation is apparently dispensable for mineral interaction [46]. To confirm this, we

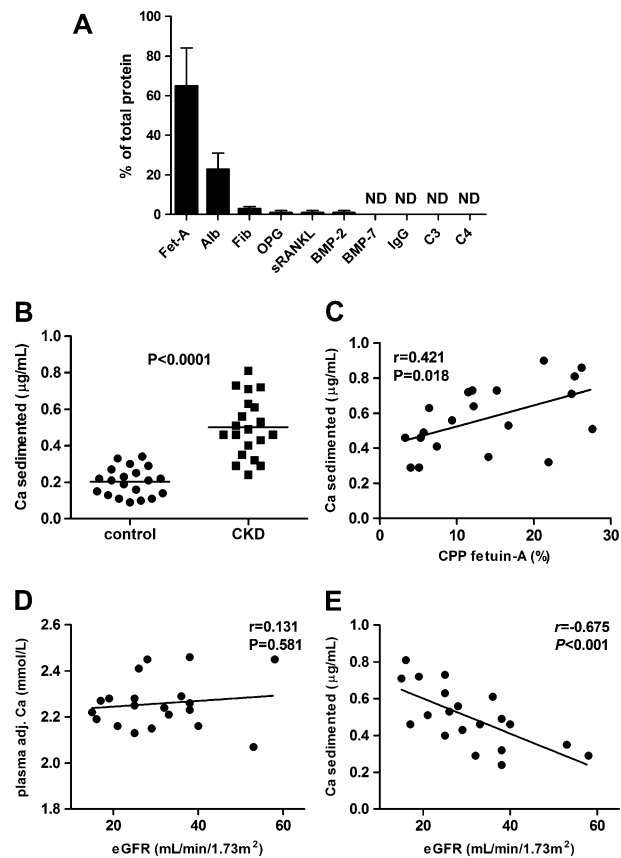


Fig. 2. Biochemical characterization of serum CPP. (A) Specific proteins were measured by immunoassay of EDTA-dissolved CPP. Protein abundance was expressed as a percentage of the total protein content ($n = 15$, mean \pm SD). Fet-A, fetuin-A; Alb, albumin; Fib, fibrinogen; sRANKL, receptor activator of nuclear factor kappa-B ligand. (B) Calcium sedimented by high-speed centrifugation from control ($n = 20$) and CKD ($n = 20$) serum. (C) Correlation between CPP-A fetuin-A levels and calcium sedimented from CKD patient serum ($n = 20$). (D) Lack of association between plasma adjusted calcium concentration and eGFR but significant inverse correlation between CPP calcium content and eGFR in patients with CKD (E).

assessed the ability of purified dephosphorylated human fetuin-A to inhibit precipitation of 5 mM calcium and phosphate in solution using the methodology of Price *et al.* [45]. Consistent with the findings of Schinke *et al.* [46], fetuin-A phosphorylation does not appear to affect its ability to inhibit mineral precipitation *in vitro* (see Figure 3B). However, since the capacity of fetuin-A to inhibit mineral precipitation does not solely reside in the formation of CPP, as monomeric fetuin-A also stabilizes calcium phosphate clusters [19], this finding does not exclude the possibility that fetuin-A phosphorylation is still needed for CPP formation or tethering. To address this, we compared the CPP fetuin-A levels in native serum to those after dephosphorylation with ALP in 10 CKD patients. Dephosphorylation consistently yielded a small (~20%) but significant reduction in CPP fetuin-A levels after centrifugation at 24 000 *g* (see Figure 4A). Due to the stable nature of CPP, we speculated that a proportion of the fetuin-A phosphorylation sites may be on the interior of the particles and are therefore inaccessible to ALP. Indeed, phos-tag SDS-PAGE analysis showed only partial (~25%) CPP fetuin-A

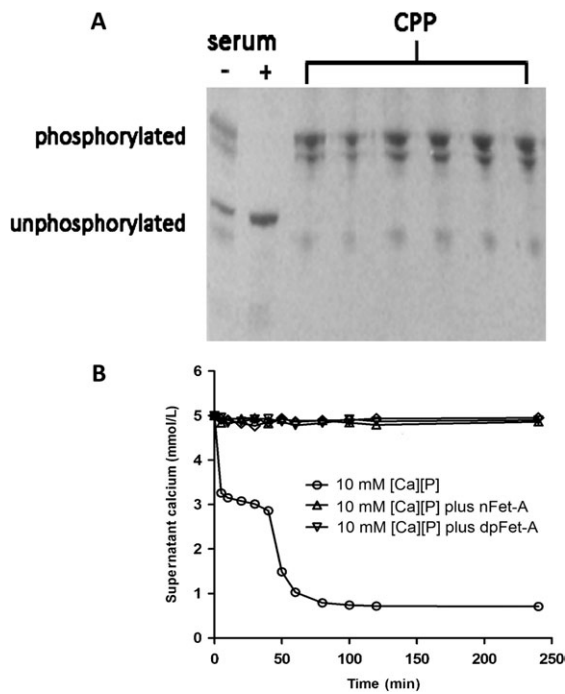


Fig. 3. Phosphorylation of fetuin-A in CPP isolated from CKD serum. **(A)** Phosphofetuin-A isoforms present in human serum and CPP of CKD patients. Western blot of serum (Lanes 1–2) and CPP fetuin-A phosphoisoforms (Lanes 3–8) separated by phosphate-affinity (phos-tag) SDS-PAGE. Enzymatically dephosphorylated serum (+) was run alongside the native sample to confirm positioning of unphosphorylated form (fastest migrating band). Serum fetuin-A is predominantly unphosphorylated but is also present in two discrete phosphorylated states. CPP fetuin-A is predominantly phosphorylated. Further control data is provided as Supplementary material. **(B)** Precipitation inhibition study using purified native fetuin-A (partially phosphorylated, nFet-A) and desphosphorylated fetuin-A (dpFet-A). Based on the method of Price *et al.* [45], 0.25 mL aliquots of solutions containing 10 mM calcium or 10 mM phosphate in 0.2 M HEPES (pH 7.4) were mixed rapidly without other additives or in the presence of 5 g/L nFet-A or dpFet-A. Fifty microlitre aliquots were taken at the time points shown, immediately spun at maximum speed in a microcentrifuge for 15 s and the supernatant diluted 1:10 with HEPES buffer to arrest further precipitation. Calcium concentration was measured by atomic absorption spectrophotometry as previously described [39].

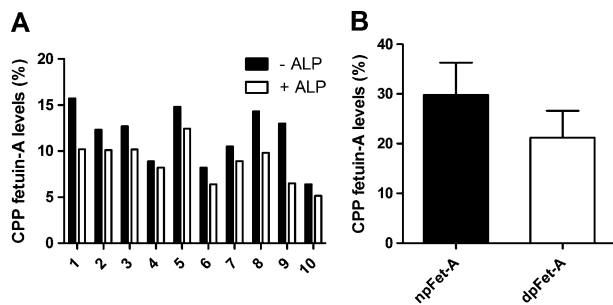


Fig. 4. Effect of CPP phosphorylation on CPP formation. **(A)** Reduction in CPP fetuin-A levels after overnight incubation of serum with 0.5 U/ μ L high activity bovine intestinal ALP (\sim 6000 U/mg protein) in 0.10 M Tris-HCl pH 9.0 containing 0.05 M MgCl₂, 0.1 mM dithiothreitol at 37°C. **(B)** Reduction in CPP fetuin-A levels in CKD serum reconstituted with highly purified dephosphorylated fetuin-A (dpFet-A) compared to native partially phosphorylated fetuin-A (npFet-A).

dephosphorylation (data not shown). We therefore devised an experiment whereby fetuin-A was depleted from pooled CKD serum and then reconstituted to physiological concentration with either native (partially phosphorylated fetuin-A \sim 15%) or enzymatically dephosphorylated fetuin-A. CPP formation was triggered by mixing reconstituted serum with a buffered salt solution containing 10 mM CaCl₂ and 6 mM Na₂HPO₄. CPP fetuin-A levels from serum reconstituted with dephosphorylated fetuin-A were \sim 30% lower than those derived from native fetuin-A (see Figure 4B). Fetuin-A phosphorylation would therefore appear to have some role in CPP tethering and formation.

Baseline clinical characteristics of study population

Two hundred patients with Stages 3 and 4 CKD were recruited to the clinical study. This was an elderly (mean age 69 \pm 11 years), mainly male (72%) and hypertensive [mean systolic blood pressure (SBP)/diastolic blood pressure (DBP) 151 \pm 22/81 \pm 11 mmHg] cohort. The mean eGFR was 33 \pm 11 mL/min/1.73m², 26% were diabetic and 44% had pre-existing CV co-morbidity. On average, more than two anti-hypertensive medications were taken per patient. Underlying causes of renal failure were 34% hypertension, 6% diabetic nephropathy, 13.5% chronic glomerulonephritis, 5% vasculitis, 6% interstitial nephritis, 5.5% cystic kidney disease, 30% obstructive or congenital disease and 30% unknown.

In this cohort, mineral metabolism appeared well controlled, with the majority of patients with adjusted plasma calcium (91%, mean 2.29 \pm 0.11 mmol/L) and phosphate concentrations (82%, 1.08 \pm 0.20 mmol/L) within the local reference range. Similarly, plasma intact PTH concentrations were less than twice the upper limit of the local normal range (<65 ng/L) in 79% of patients. Only 10 (5%) patients were receiving calcium-based phosphate binders, 3 (1.5%) were taking non-calcium-based binders and 16 (8%) were receiving 1(OH) vitamin D₃.

Serum biomarker levels in CKD and control groups

Compared to age-matched healthy controls [mean age 67.8 \pm 6.0, 63.9% male, median body mass index 26.9 (23.2–31.7) kg/m²], serum inflammatory markers (hs-CRP, IL-6, TNF- α), oxLDL, BMP-2 and BMP-2/BMP-7 ratio were all significantly higher in the CKD group, while serum BMP-7 concentrations were significantly lower. CPP fetuin-A was undetectable in control sera (Table 1).

Factors independently associated with CPP fetuin-A levels

The relationship between tertiles of CPP fetuin-A levels and baseline clinical and biochemical parameters is shown in Table 2. After adjustment for all baseline variables that were significant in univariate analysis (P < 0.1), CPP fetuin-A levels were significantly associated with plasma phosphate concentration (B = 4.504, P = 0.024), hs-CRP (B = 3.044, P = 0.001), oxLDL (B = 3.095, P = 0.040), CV co-morbidity (B = 2.456, P = 0.002) and inversely associated with eGFR (B = -0.237, P = 0.001). This model

explained 51% of the variation in CPP fetuin-A levels. When log BMP-2 and log BMP-7 were forced into the model, hs-CRP, oxLDL and eGFR lost significance but the explained variation in CPP fetuin-A remained unchanged ($R^2 = 0.497$).

In serial analysis ($n = 105$), the change in CPP fetuin-A levels >12 months was significantly correlated with the change in serum hs-CRP concentration ($r = 0.362$, $P < 0.001$) and change in eGFR ($r = -0.341$, $P < 0.001$).

In order to assess whether CPP fetuin-A levels were related to bone turnover, we measured a specific serum marker of Type I collagen degradation, β -CTx. Serum β -CTx measurements were available in 80 patients (mean age 68.2 ± 8.9 , 65% male, mean eGFR $33 \text{ mL/min/1.73m}^2$)

and were significantly higher in females compared to males (mean $1159 \pm 286 \text{ ng/L}$, mean difference 102 ng/L , $P = 0.007$). β -CTx was significantly correlated with CPP fetuin-A levels ($r = 0.311$, $P = 0.002$) and inversely associated with eGFR ($r = -0.180$, $P = 0.010$). Controlling for eGFR, CPP fetuin-A levels remained significantly associated with serum β -CTx concentration ($r_{\text{partial}} = 0.245$, $P = 0.005$).

Factors independently associated with serum BMP levels

Baseline correlations of serum BMP-2 and BMP-7 are given in Table 3. In multivariate analysis, only history of pre-existing CV co-morbidity, hs-CRP and BMP-7 concentration remained significantly associated with serum

Table 1. Comparison of serum biomarker levels in CKD patient and control groups^a

	Stage 3/4 CKD ($n = 200$)	Control ($n = 78$)	P-value
CPP fetuin-A levels (%)	12.1 ± 7.8	Undetectable ^b	NA
hs-CRP (mg/L)	$2.3 (0.9, 5.8)$	$1.3 (0.5, 2.7)$	0.010
IL-6 (ng/L)	$6.5 (4.5, 9.5)$	$4.6 (2.9, 6.1)$	0.014
TNF- α (ng/L)	$16.7 (12.6, 21.9)$	$4.6 (2.9, 5.3)$	<0.001
oxLDL (U/L)	$67.3 (45.9, 130.0)$	$51.8 (25.0, 96.1)$	0.008
BMP-2 (ng/L)	$136.5 (105.8, 195.5)$	$101.5 (66.9, 139.3)$	0.004
BMP-7 (ng/L)	$14.1 (9.0, 19.1)$	$22.7 (12.2, 45.6)$	0.001
BMP-2/BMP-7 ratio	$11.41 (7.34, 16.04)$	$5.77 (3.94, 11.52)$	<0.001

^aData are mean \pm SD or median (25, 75th percentile). NA, not applicable.

^bUndetectable—no significant difference in fetuin-A concentration was observed before and after centrifugation at $24 \text{ } 000 \text{ g}$.

Table 2. Baseline characteristics by tertile of CPP fetuin-A level^a

Variable	Low ($\leq 6.7\%$; $n = 66$)	Intermediate (6.8–14.2%; $n = 67$)	High ($\geq 14.3\%$; $n = 67$)	P-value for trend
CPP fetuin-A (%)	4.4 ± 1.2	10.5 ± 2.2	21.1 ± 5.6	NA
Gender (% male)	76	67	73	0.527
Age (year)	65.2 ± 11.1	69.3 ± 11.8	72.0 ± 10.1	0.002
History of CVD (%)	36	43	52	0.062
History of diabetes (%)	27	21	28	0.564
BMI (kg/m^2)	$28.2 (25.4, 33.6)$	$28.7 (24.8, 33.3)$	$28.4 (25.8, 34.1)$	0.658
eGFR (mL/min/1.73m^2)	38.9 ± 11.1	32.8 ± 8.2	26.5 ± 8.7	<0.001
Systolic BP (mmHg)	149 ± 21	152 ± 20	152 ± 24	0.659
Diastolic BP (mmHg)	82 ± 12	82 ± 11	80 ± 11	0.602
Heart rate (b.p.m.)	70 ± 11	70 ± 12	72 ± 14	0.532
APWV (m/s)	12.2 ± 2.4	12.9 ± 2.8	13.9 ± 2.3	0.001
ACEi/ARB use (%)	79	60	57	0.015
CCB use (%)	44	46	48	0.906
Statin use (%)	64	55	60	0.613
Alcohol intake (U/week)	6.6 ± 8.1	8.5 ± 11.6	7.4 ± 8.5	0.513
Smoking (packs/year)	13.7 ± 21.2	15.7 ± 22.1	23.4 ± 33.8	0.081
Haemoglobin (g/dL)	13.0 ± 1.6	12.6 ± 1.7	12.4 ± 1.6	0.171
Albuminuria (mg/mmol)	$3.4 (1.0, 21.8)$	$5.4 (1.4, 35.7)$	$6.9 (2.9, 45.7)$	0.045
Plasma albumin (g/L)	43 ± 3	42 ± 3	43 ± 3	0.413
Plasma-adjusted calcium (mmol/L)	2.29 ± 0.11	2.29 ± 0.12	2.29 ± 0.12	0.993
Plasma phosphate (mmol/L)	1.04 ± 0.19	1.07 ± 0.18	1.13 ± 0.22	0.043
Plasma iPTH (ng/L)	$65 (46, 99)$	$74 (50, 95)$	$108 (62, 162)$	<0.001
Plasma total cholesterol (mmol/L)	4.28 ± 1.39	4.18 ± 1.27	4.15 ± 1.01	0.829
Serum hs-CRP (mg/L)	$1.12 (0.60, 2.92)$	$2.40 (1.20, 4.68)$	$5.10 (1.80, 9.90)$	<0.001
Serum IL-6 (ng/L)	$6.0 (3.8, 8.0)$	$6.4 (4.5, 9.5)$	$7.9 (5.3, 10.3)$	0.021
Serum TNF- α (ng/L)	$15.6 (12.7, 18.1)$	$17.9 (12.5, 22.9)$	$16.1 (12.3, 22.2)$	0.165
Serum oxLDL (U/L)	$60.7 (37.3, 124.7)$	$60.7 (45.9, 124.0)$	$96.0 (58.6, 138.6)$	0.048
Serum BMP-2 (ng/L)	$134.2 (100.5, 171.5)$	$127.6 (102.1, 198.8)$	$143.0 (114.2, 210.3)$	0.162
Serum BMP-7 (ng/L)	$18.5 (15.5, 22.7)$	$14.5 (9.2, 20.0)$	$8.7 (7.2, 11.3)$	<0.001
Serum BMP-2/BMP-7 ratio	7.43 ± 2.71	11.14 ± 4.35	18.06 ± 4.94	<0.001

^aData are mean \pm SD or median (25, 75th percentile). ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; BP, blood pressure; CCB, calcium channel blocker; CVD, cardiovascular disease; iPTH, intact parathyroid hormone; NA, not applicable.

BMP-2 after adjustment for other variables (model $R^2 = 0.432$), while eGFR, age, hs-CRP, oxLDL and BMP-2 all remained significantly associated with serum BMP-7 concentration (model $R^2 = 0.467$).

Independent association between CPP fetuin-A levels and APWV

Higher CPP fetuin-A levels were associated with increased APWV (Table 4). This was maintained after adjustment for age, SBP, heart rate, eGFR and hs-CRP ($B = 0.059$, $P = 0.016$, $R^2 = 0.362$). CPP fetuin-A levels were also significantly correlated with C-terminal fibroblast growth factor-23 (FGF-23; $r = 0.301$, $P < 0.001$) and OPG concentration ($r = 0.198$, $P = 0.005$). We have previously shown plasma OPG to be independently associated with APWV in this cohort [41]. CPP fetuin-A remained significantly associated with APWV when plasma OPG concentration was added into the model with the other aforementioned covariates (data not shown). On the other hand, significant association between CPP fetuin-A and APWV was lost

when serum BMP-2 and BMP-7 concentration were added into the model ($P = 0.081$).

Discussion

In this study, we have used centrifugation and ultrafiltration techniques to confirm the presence of fetuin-A-containing CPPs in the serum of pre-dialysis patients. This extends previous studies showing fetuin-A-containing CPP (or FMC) in serum from patients undergoing haemodialysis [21], serum from rats treated with etidronate [47], vitamin D [48] and in the peritoneal dialysate of patients with advanced CKD [49]. Furthermore, consistent with the report from Heiss *et al.* [49], we found that serum CPP to be composed mainly of fetuin-A, albumin and calcium phosphate, with trace amounts of fibrinogen, also reported by Hamano *et al.* [21]. Structural and biophysical aspects of these particles and their formation have been comprehensively published elsewhere [18, 19, 49, 50].

Importantly, we show for the first time in human serum that only phosphorylated fetuin-A is present in these high-molecular-weight (HMW) mineral complexes. This is consistent with the recent work of Matsui *et al.* [38] in a rat model of renal failure, where only fully phosphorylated fetuin-A was found in circulating mineral particles. Fetuin-A phosphorylation is thought to be necessary for inhibition of the insulin receptor [51], but the functional significance (if any) of fetuin-A phosphorylation in CPP is presently undetermined. Although we show that fetuin-A phosphorylation does not appear essential for inhibition of mineral precipitation by monomeric fetuin-A, and therefore consistent with the findings of other studies [46], we also present preliminary data which suggest that fetuin-A phosphorylation may be necessary for CPP formation and tethering. This does not appear to be an absolute requirement, however, as CPPs were still formed from serum reconstituted with completely dephosphorylated fetuin-A. Whether these particles differ in their stability or other physical characteristics requires further analysis. Alternatively, we speculate that fetuin-A phosphorylation may serve as a signal for receptor-mediated clearance and further work is underway to examine this hypothesis.

Phosphate-affinity mobility shift electrophoresis revealed two distinct phosphorylated isoforms of human fetuin-A. Haglund *et al.* [52] reported that human plasma fetuin-A was phosphorylated at two sites, Serine-120 (within the mature heterodimeric protein) and Serine-312 (within the single-chain precursor). However, recent analysis of the human serum phosphoproteome suggests that other serine residues may also be phosphorylated [53]. It is presently unclear which specific phosphorylated species each discrete band represents and their individual significance.

Hamano *et al.* [21] were the first to suggest that CPP fetuin-A levels rather than serum fetuin-A may provide a better indication of extra-osseous calcification stress in CKD patients. Indeed, in their study of 73 CKD patients (43 with Stages 3–4 CKD), CPP fetuin-A but not total serum fetuin-A levels were significantly correlated with coronary artery calcification scores. This underscores our view that it is vital to consider the structural and functional

Table 3. Baseline correlations with serum BMP-2 and BMP-7 concentration^a

	BMP-2		BMP-7	
	<i>r</i>	P-value	<i>r</i>	P-value
Age	0.118	0.095	-0.194	0.006 ^b
Gender (male)	-0.009	0.902	-0.136	0.055
eGFR	-0.075	0.292	0.359	<0.001 ^b
Diabetic status	0.086	0.227	-0.009	0.896
Pre-existing CVD	0.272	<0.001 ^b	-0.135	0.037
Adjusted plasma calcium	-0.052	0.465	0.074	0.300
Plasma phosphate	0.081	0.253	-0.231	0.018 ^b
Plasma iPTH ^c	0.053	0.458	-0.204	0.004
Plasma albumin	0.102	0.149	0.197	0.005
Serum hs-CRP ^c	0.165	0.020 ^b	-0.283	<0.001 ^b
Serum IL-6 ^c	0.127	0.047	0.068	0.049
Serum TNF- α ^c	0.049	0.491	0.037	0.608
Serum oxLDL ^c	0.206	0.035	-0.123	0.082
Serum BMP-2 ^c	NA	NA	0.412	<0.001 ^b
Serum BMP-7 ^c	0.412	<0.001 ^b	NA	NA

^aCVD, cardiovascular disease; iPTH, intact parathyroid hormone; NA, not applicable.

^bIndicates variables the remained significantly associated after adjustment for all other variables.

^cLog transformed.

Table 4. Multivariate adjustment of the association between CPP fetuin-A levels and APWV^a

Model	
1. Crude	0.110** (0.060 to 0.150)
2. 1 + age (years)	0.066** (0.024 to 0.108)
3. 2 + systolic BP and heart rate	0.065** (0.020 to 0.110)
4. 3 + eGFR (mL/min/1.73m ²)	0.065** (0.019 to 0.110)
5. 4 + hs-CRP (mg/L) ^b	0.058* (0.011 to 0.105)
6. 5 + BMP-2/BMP-7 ratio	0.028** (-0.020 to 0.065)

^a $n = 185$; unstandardized B-coefficients (95% CIs).

^bLog transformed.

** $P < 0.001$.

* $P < 0.01$.

heterogeneity of the fetuin-A pool and the ability of different methods to detect them [54]. In our present study, serum (total fetuin-A) concentration and CPP fetuin-A levels were not significantly correlated ($r = 0.006$, $P = 0.936$). It is critical to emphasize, however, that there are several key differences between these studies. Firstly, their study used an alternative commercial fetuin-A ELISA (Epitope Diagnostics Inc.) to this study, which we have shown in this study and previously [28] to show poor agreement with the kit used in our study (Biovendor). Secondly, we used a modified centrifugation protocol to isolate a greater proportion of circulating HMW fetuin-A complexes. Thirdly, we assessed APWV and not intimal coronary calcification. The pathogenesis and sequelae of increased APWV and coronary calcification are different: increased APWV is generally related to changes in the tunica media, leading to left ventricular afterload, left ventricular hypertrophy and a reduction in coronary perfusion; whereas coronary artery calcification is largely intimal and related to atherosclerotic burden and plaque stability. Although we hypothesized that increased aortic stiffness may be a result of increased medial calcification and hence reflected by CPP fetuin-A levels, it is also possible that the procalcific environment observed in our patients is intimately associated with other interrelated arterial changes that are directly responsible for the loss of vessel compliance rather than calcification *per se*.

We previously found no significant relationship between total serum fetuin-A concentration and baseline APWV [16]. However, in this study, we found that higher CPP fetuin-A levels were independently associated with increased APWV. APWV is a composite marker that reflects both arterial medial calcification and other structural changes to elastin and collagen within the arterial wall [55]. Therefore, the independent relationship with APWV suggests that CPP fetuin-A levels may be more directly associated with the pathological process of arterial medial calcification. Since reductions in CPP fetuin-A levels appear to precede changes in arterial calcification scores in patients treated for secondary hyperparathyroidism [21], and given that appearance of CPP precede the development of vascular calcification in uraemic mice [38], this fraction of the fetuin-A pool may well represent mineralization risk and provide a measure of the pressure under which the bodies calcification inhibitory systems are being placed.

In multivariate modelling, hs-CRP, oxLDL and plasma phosphate concentration were all independently associated with CPP fetuin-A levels. Importantly, all three stimuli have been implicated in promoting the switch of VSMC to an osteochondrocytic-like phenotype and may help explain further the link between inflammation and vascular calcification.

Compared to age-matched healthy controls, serum hs-CRP, IL-6 and TNF- α concentrations were significantly higher in the CKD group. There is now increasing evidence that inflammatory cytokine networks modulate the CV risk in this patient group [22], and there appears to be an interrelation between mineral imbalance (hyperphosphataemia in particular) and inflammation pathways [56]. Recent work suggests that TNF- α may increase Msx2 expression (a bone-related transcription factor) via the NF- κ B path-

way, in regulating ALP expression, thereby promoting mineralization [57]. The lack of relationship between CPP fetuin-A levels and TNF- α concentration in the present study probably reflects the fact that these cytokines are produced locally within the vasculature and largely act in a paracrine manner. Systemic markers of inflammation like C-reactive protein may therefore represent a better indication of this activity. As previously noted, increased oxLDL can also promote vascular calcification via enhanced TNF- α secretion and mitogen-activated protein kinase (MAPK) signalling [33, 34]. Similarly, phosphate appears to have a central role in driving the osteogenic differentiation and apoptosis of VMSC [58]. Although mineral parameters were generally within conventional reference ranges, changes in phosphate and FGF-23 metabolism begin with GFRs >60 mL/min/1.73m² and have been associated with an increased incidence of CV disease [59, 60].

We also found a significant independent association between CPP fetuin-A levels and the serum BMP-2/BMP-7 ratio. Little data exist on serum levels of BMPs in similar populations. BMP-2 levels have previously been found to be increased in pooled uraemic serum. Since the effects of BMP-2 and BMP-7 on VSMC are largely antagonistic, we hypothesized that the ratio of BMP-2/BMP-7 may be more informative than single BMP measurements alone. Indeed, baseline CPP fetuin-A levels were not significantly associated with BMP-2 concentration but were highly significantly associated with the BMP-2/BMP-7 ratio. Again, compared to healthy controls, this ratio was significantly higher in CKD patients. Dalfino *et al.* [61] have previously postulated that BMP-2 may serve as the link between oxidative stress and vascular stiffness (using brachial-ankle PWV) in CKD. In support of this, we found a highly significant and independent association between BMP-2/BMP-7 ratio (but not BMP-2 alone) and APWV, the 'gold standard' measure of arterial stiffness.

Interestingly, the uptake of synthetic octacalcium phosphate nanocrystals by murine smooth muscle cells has been shown to induce BMP-2 expression [32]. Whether the uptake of fetuin-A-bound mineral *in vivo* has similar effects on gene expression and VSMC phenotype remains to be tested. It is tempting to speculate that stabilization of small calcium phosphate complexes by fetuin-A may render them less osteogenic. The uptake of basic calcium phosphate nanocrystals (<2 μ m in diameter) by macrophage is pro-inflammatory, activating protein kinase C (PKC) and MAPK pathways leading to TNF- α production [62, 63]. Critically therefore, the proposed 'mineral chaperone' function provided by fetuin-A [20] may minimize inflammatory risk and facilitate 'safe' clearance.

The ability of fetuin-A to interact with mineral resides in its amino terminal cystatin-like domain D1, through an array of acidic residues on an extended β -sheet [18]. Both monomeric and higher-order particles interact with mineral, and both act to stabilize supersaturated mineral solutions (like serum) [19]. Thus, CPP fetuin-A is not the only fraction of interest and perhaps, a functional test of total inhibitory capacity has greater potential as recently described by Ismail *et al.* [64].

The origins of circulating CPP are also unclear. Current evidence suggests that they mainly originate from the bone compartment. Consistent with this, Hamano *et al.* reported a reduction in CPP fetuin-A levels after treatment with the calcimimetic, cinacalcet, concomitant with a reduction in PTH [21]. In this study, we found a highly significant correlation between CPP fetuin-A levels and serum β -CTX concentration, a highly specific marker for Type I collagen degradation in bone. Indeed, further work from Price *et al.* suggests that fetuin-A regulates the mineralization of bone outside of the collagen matrix [65].

This study has several limitations that we acknowledge. Firstly, this is an epidemiological study and does not infer causality or directionality in these relationships. Secondly, we have also made no direct assessment of arterial calcification burden or bone mineral density. Instead, we have measured arterial stiffness, one of the pathophysiological consequences of medial calcification. Finally, the findings of this study are largely based on single one-off measurements and we do not explore the significance of temporal changes in these markers.

In conclusion, we have shown here for the first time that only phosphorylated fetuin-A is present in the CPP of pre-dialysis CKD patients and that CPP fetuin-A levels are independently associated with serum inflammatory markers, BMP-2/BMP-7 ratio and phosphate concentration. In multivariate analysis, CPP fetuin-A levels were independently associated with aortic stiffness.

Supplementary data

Supplementary data are available online at <http://ndt.oxfordjournals.org>.

Acknowledgements. This study was supported by the Department of Renal Medicine and the Clinical Investigation Research Unit at BSUH NHS Trust.

Conflict of interest statement. None declared.

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Received for publication: 22.7.11; Accepted in revised form: 14.9.11