SUPPLEMENTAL MATERIAL

Complete Methods

Study Design and Population

The ACADEMIC study was a single-center, prospective, observational study examining aspects of cardiovascular risk in a cohort of 200 individuals with Stages 3 and 4 CKD.[1, 2] These patients were recruited from nephrology outpatient clinics at Brighton and Sussex University Hospitals NHS Trust between March 2006 and September 2009. Patient demographics, clinical measurements and full medical history were recorded at entry to the study. Exclusion criteria included a previous diagnosis of left ventricular failure with left ventricular ejection fraction less than 35%, aortic stenosis with gradient >30 mmHg, atrial fibrillation with ventricular rate greater than 100 beats per minute and age less than 40 years or greater than 90 years. All participants were treated with the aim of achieving United Kingdom Renal Association targets for management of blood pressure in CKD at the time of their participation in the study. The choice of antihypertensive medication remained at the discretion of the patient's clinician but generally followed British Hypertension Society guidelines.[3] Calcium-based phosphate binder use was defined as the equivalent intake of elemental calcium of ≥1 g per day. Relevant medical history was self-reported by the participants or accessed from records held by their General Practitioner. Pre-existing cardiovascular co-morbidity was defined as a history of transient ischemic attack, stroke, myocardial infarction, angina or if the patient had undergone treatment for cardiovascular disease (e.g. coronary artery bypass grafting or angioplasty).

Patients were followed prospectively until they died, started renal replacement therapy or the observation period ended. Random, non-fasting, plasma, serum and plain urine samples were collected at entry to the study and at bi-annual follow-up visits, concomitant with clinical and vascular assessments. Due to limited availability of stored serum samples at the study enrollment visit, the present analysis is restricted to the participants who had samples available for measurement of serum calcification risk factors at the 6 month visit. We used

clinical and laboratory data collected at this 6-month visit as co-variates in all subsequent analyses. Of these 184 individuals, 181 were Caucasian, 2 Arab, and 1 Black African.

Participants gave written informed consent, and the study was approved by the West Sussex Research Ethics Committee [REC Ref.# 05/Q1911/89] and conducted in accordance with the Declaration of Helsinki.

Vascular Assessments

All vascular measurements were conducted in a quiet, temperature-controlled room. Patients were requested to refrain from smoking and ingesting caffeine prior to the assessment but were otherwise unrestricted. Oscillometric blood pressure was measured twice using an appropriate cuff size with the patient supine after 5 and 10 minutes of rest (Omron 705 CP, Tokyo, Japan). The mean of the two recordings of systolic BP (SBP) and diastolic BP (DBP) was recorded. Mean arterial pressure (MAP) was determined as: DBP + ((SBP –DBP) /3). APWV measurement was performed using Complior™ (Colson, Les Lilas, France) according to best practice guidelines. Dedicated mechanotransducers were directly applied to the skin overlying the carotid and femoral arteries and the distance between the two sites was measured. The transit time was determined by a correlation algorithm between each simultaneous recorded wave and PWV was obtained using the following equation: PWV=distance/time. The validation and reproducibility of this method have been previously published.[4] Measurement of APWV was performed by only three trained observers throughout the study and a repeatability study demonstrated no significant inter-observer variability. Final APWV readings were based on the mean of two measurements. The progression of aortic stiffening (ΔAPWV) was evaluated in each individual with APWV readings at 4 consecutive study visits (2.5 years) from baseline using linear mixed modelling. Progression was not evaluated in the remaining 91 individuals due to attainment of the study endpoint (death or dialysis) within this time (n=43), completion of the study observation period before 2.5 years follow-up (n=33), major vascular surgery (n=3), undetectable pulse waveform (n=8) or impalpable pulses (n=4). APWV slopes were non-normally distributed and

qualifying patients (n=93) were categorized into two groups: those with an APWV slope ≥20% (progressors) and those with an APWV <20% (non-progressors).

Sample Collection and Biochemical Analysis

Samples taken for standard clinical practice were analysed immediately at the central BSUH pathology laboratory, and additional aliquots were stored for future testing. Lithium-heparin plasma samples and clotted blood samples (gel-free) were taken using standard phlebotomy techniques, centrifuged for 10 min at 3500 *g* and stored at -70°C until batched analysis. Samples were only subjected to a single thaw at 4°C prior to analysis. Standard biochemical analysis was performed using a routine automated analyzer (Roche Modular, Haywards Heath, UK). Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) equation.[5] Adjusted calcium concentrations were calculated according to the following equation: [measured calcium, mmol/L] + 0.02 (40-[albumin, g/L]). Ionized calcium (iCa) was measured using an ion-selective electrode on an ABL 700 analyzer (Radiometer, Copenhagen, Denmark). Serum hs-CRP was measured by particle-enhanced immunonephelometry on the Dade Behring ProSpec analyzer (Siemens, Camberley, UK). Intra-assay and interassay imprecision were <3.8 and 5.2 % respectively, limit of detection was 0.175 mg/L. Plasma intact PTH (iPTH) and β-isomerized C-terminal telopeptides of Type I collagen (CTx) were measured using Elecsys reagents for the Modular Analytics E170 immunoanalyser (Roche Diagnostics, Burgess Hill, UK). Plasma pyrophosphate concentration was determined colorimetrically after ultracentrifugation, concentration and separation from orthophosphate and phosphate esters as previously described by Heinonen *et al.*[6] Serum TNF-α was measured using a commercially available ELISA kit (R&D Systems, Abbindgon, UK). Between-batch imprecision was 5.7% at 10.5 pg/mL and the limit of detection was 0.1 pg/mL. Samples for TNF-α analysis were measured in duplicate and mean value recorded. Serum magnesium concentrations were determined in triplicate using the xylidylblue method.[7]

Serum Calcification Propensity Test

The serum calcification propensity test was performed using a Nephelostar nephelometer (BMG Labtech, Offenburg, Germany) as previously described.[8] All serum samples were measured in a blinded manner at the University Hospital Bern, Switzerland, Dept. Nephrology, Hypertension and Clinical Pharmacology. Briefly, three solutions were prepared: solution 1: 140 mM NaCl; solution 2: 40 mM $CaCl_2$ + 100 mM Hepes + 140 mM NaCl pH-adjusted with 10 M NaOH to 7.40 at 37°C, solution 3: 19.44 mM Na_2HPO_4 + 4.56 mM NaH_2PO_4 + 100 mM Hepes + 140 mM NaCl pH-adjusted with 10 M NaOH to 7.40 at 37°C. All chemicals were analytical grade and purchased from AppliChem (Darmstadt, Germany). Pipetting steps were performed in 96-well plates in a thermo-constant room. The order was as follows: (1) NaCl solution: 20 µl/well, (2) serum: 80 µl/well, (3) phosphate solution: 50 µl/well, (4) calcium solution: 50 µl/well. Measurements were performed in a thermo-constant room with an internal temperature of the Nephelostar device of 36.5°C to 37°C. The assay was performed for 200 cycles with 1.5-seconds measurement time per well and a position delay of 0.1 seconds. The total assay run time was 10 hours. Data were then processed by calculating the precipitation time T_{50} from nonlinear regression curves. Samples were measured in triplicate. The analytical coefficient of variation of a pooled serum precipitating at 270 min. was 8.3%.

Fetuin-A Measurements

Serum fetuin-A was measured by ELISA (Biovendor, Brno, Czech Republic) as previously described.[9] Briefly, total serum fetuin-A (total Fet-A) concentration was measured following centrifugation of clotted blood samples (10 min, 2,000x*g* 4°C). Aliquots of each serum sample were then subjected to further centrifugation at 24,000xg for 2 h at 4°C in sealed tubes, and the supernatant re-analyzed for fetuin-A using the same ELISA assay. For total serum Fet-A measurements samples were diluted 1:10,000 in dilution buffer as recommended by the manufacturer. Supernatants were assayed after 1:8500 dilution in the same buffer. CPP-associated fetuin-A concentrations (CPP Fet-A) were then calculated by

the difference in total serum fetuin-A and supernatant monomeric fetuin-A concentrations (mono Fet-A): CPP Fet-A = total Fet-A - mono Fet-A. Between-batch imprecision was 2.6% at 30 mg/L and the limit of detection was 1.1 mg/L. All measurements were made in triplicate. Samples with a triplicate CV >3.5% were re-analyzed. Sample dilutions, reagent additions, incubations and photomeric readings were performed using an automated DS2 ELISA processing system equipped with disposable tips (Dynex, Chantilly, VA, USA). The limit of quantitation for CPP Fet-A estimation was 7.5 mg/L. For the purposes of analysis, samples yielding measurements below this limit (n=14) were assigned a concentration of 7.5 mg/L.

Exposures and Outcomes

The primary exposure was baseline serum T_{50} and the secondary exposure was CPP Fet-A concentration. The outcome measure for this analysis was time to death from any cause that occurred after the 6-month follow-up visit and before censoring in 01 Nov 2012. Survival data was gathered prospectively during the study. The survival status of patients was then confirmed using electronic hospital computer records.

Statistical Analysis

Demographic, cardiovascular and biochemical factors were compared across tertiles of baseline serum T_{50} using one-way ANOVA, Kruskal-Wallis test and χ^2 tests as appropriate. PTH, CTx, hsCRP and TNF-α concentrations showed a skewed distribution and were natural-log transformed before further analysis. Determinants of baseline serum T_{50} were evaluated using linear regression models. Continuous baseline predictors were standardized and expressed per SD increase. Since clinical correlates of serum T_{50} have not be previously defined, all those co-variates with a P value <0.1 in univariate analysis were entered simultaneously into the final multivariable model. Models were tested for collinearity using variance inflation factors and stability of the regression coefficients. We observed significant co-linearity between total and mono fet-A, and between total and ionized calcium concentrations and were modelled separately. The association between baseline APWV and

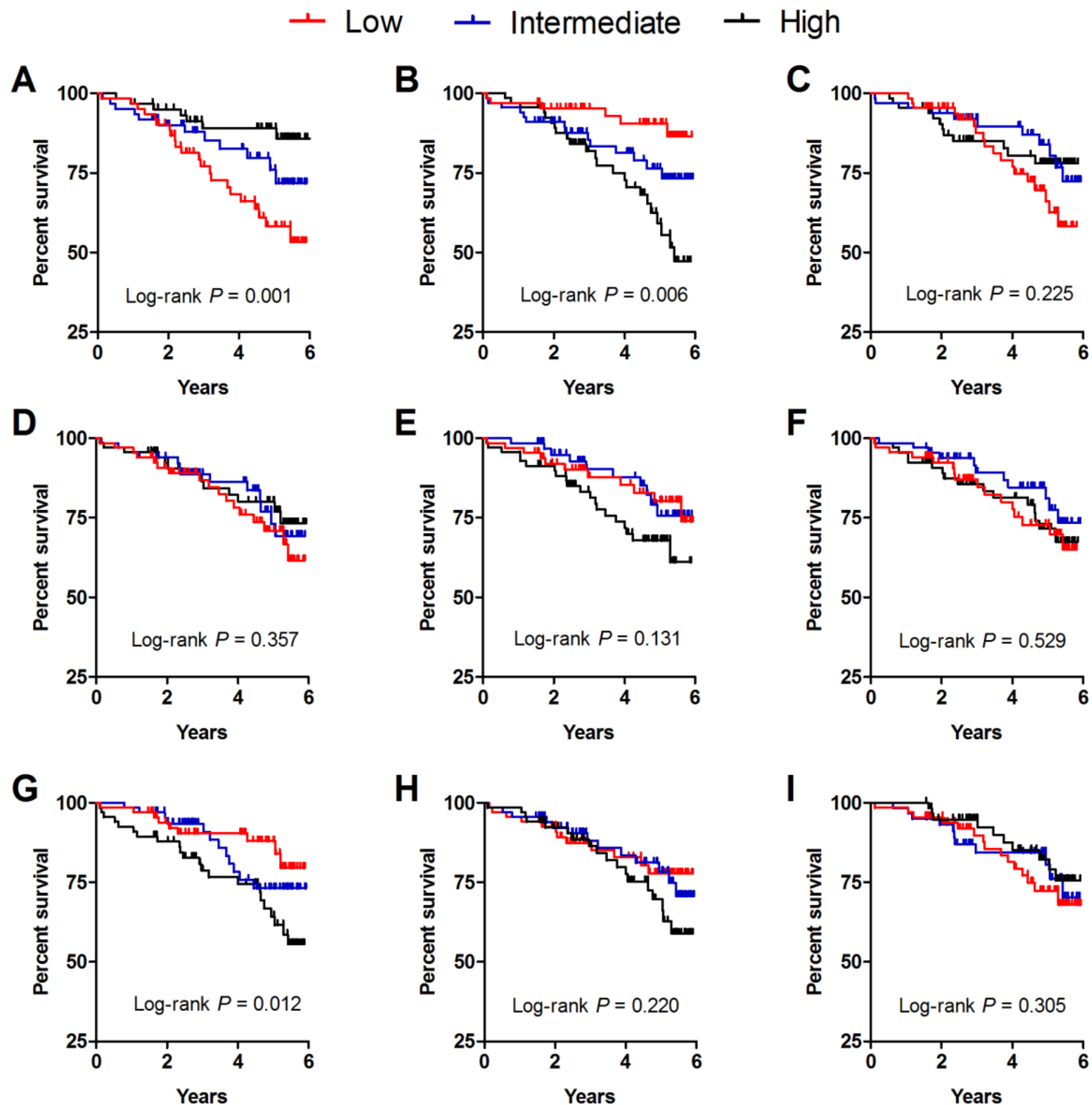
serum T_{50} was analysed using multiple linear regression, adjusting for known determinants of APWW: age, eGFR, MAP and heart rate.[10] Multiple logistic regression was used to evaluate the relationship between APWV progression (dichotomized into < or ≥20% increase from baseline over 30 months) and baseline serum T_{50}, adjusting for the above pre-specified co-variates, and other factors significantly associated with APWV progression in univariate analysis: baseline APWV, hsCRP, CPP Fet-A, phosphate, pyrophosphate and CTx concentrations. We analyzed the risk of all-cause death according to these exposures on a continuous scale (per SD increment) and across tertiles in order account for nonlinear effects. The Kaplan-Meier method was used to present unadjusted univariate analyses and we tested for trends with log-rank tests. After confirming the proportionality assumption using Schoenfeld residual and log-minus-log survival plots, Cox proportional hazard models were used to adjust for confounding. The multivariable modeling strategy was hierarchical, pre-specified, and consistent for both primary and secondary exposures. For analysis according to tertiles, the highest tertile was served as the reference category for serum T_{50} and the lowest tertile served as the reference group for CPP Fet-A. Five sequential sets of covariates were considered: model 1 included age and gender; model 2 included covariates from model 1 plus eGFR and proteinuria; model 3 included co-variates from model 2 plus phosphate concentration; model 4 included covariates from model 3 plus history of pre-existing CVD, SBP and smoking status; model 5 included covariates from model 4 plus previously identified determinants of each exposure: for serum T_{50} analyses were further adjusted for albumin, magnesium, pyrophosphate, ionized calcium, monomeric Fet-A and CTx; for CPP Fet-A analyses were adjusted for hsCRP concentration. In separate analyses, exclusion of those patients receving calcium supplementation did not significantly affect the modeling (data not shown). Potential modifiers of the association between serum T_{50} and mortality by selected baseline confounders were evaluated by introducing interaction terms into the model and tested by the likelihood ratio test. The area under the receiver operating characteristic curve (AUC) was calculated to compare prognostic value of each exposure using the non-parametric method of DeLong.[11] Variance components (within-subject, σ^2_w and between-

subject, σ^2_b variance) were estimated using random-effects ANOVA models and as previously described and intraclass correlation coefficients (ICC) were derived using the following formula: $\sigma^2_b/(\sigma^2_b + \sigma^2_w)$.[12] All analyses were performed using Stata release 12/IC (College Station, TX, USA) and two-sided values of P<0.05 were considered statistically significant.

References

1. Ford, ML, Tomlinson, LA, Chapman, TP, Rajkumar, C, Holt, SG: Aortic stiffness is independently associated with rate of renal function decline in chronic kidney disease stages 3 and 4. *Hypertension,* 55**:** 1110-1115, 2010.
2. Smith, ER, Tomlinson, LA, Ford, ML, McMahon, LP, Rajkumar, C, Holt, SG: Elastin degradation is associated with progressive aortic stiffening and all-cause mortality in predialysis chronic kidney disease. *Hypertension,* 59**:** 973-978, 2012.
3. Sever, P: New hypertension guidelines from the National Institute for Health and Clinical Excellence and the British Hypertension Society. *J Renin Angiotensin Aldosterone Syst,* 7**:** 61-63, 2006.
4. Asmar, R, Benetos, A, Topouchian, J, Laurent, P, Pannier, B, Brisac, AM, Target, R, Levy, BI: Assessment of arterial distensibility by automatic pulse wave velocity measurement. Validation and clinical application studies. *Hypertension,* 26**:** 485-490, 1995.
5. Levey, AS, Stevens, LA, Schmid, CH, Zhang, YL, Castro, AF, 3rd, Feldman, HI, Kusek, JW, Eggers, P, Van Lente, F, Greene, T, Coresh, J: A new equation to estimate glomerular filtration rate. *Ann Intern Med,* 150**:** 604-612, 2009.
6. Heinonen, JK, Honkasalo, SH, Kukko, EI: A method for the concentration and for the colorimetric determination of nanomoles of inorganic pyrophosphate. *Anal Biochem,* 117**:** 293-300, 1981.
7. Bohoun, C. Mircodetermination of magnesium in various biological media. Clin Chim Acta. 7:811-7, 1962.
8. Pasch, A, Farese, S, Graber, S, Wald, J, Richtering, W, Floege, J, Jahnen-Dechent, W: Nanoparticle-based test measures overall propensity for calcification in serum. *J Am Soc Nephrol,* 23**:** 1744-1752, 2012.
9. Smith, ER, Ford, ML, Tomlinson, LA, Rajkumar, C, McMahon, LP, Holt, SG: Phosphorylated fetuin-A-containing calciprotein particles are associated with aortic stiffness and a procalcific milieu in patients with pre-dialysis CKD. *Nephrol Dial Transplant,* 27**:** 1957-1966, 2012.
10. Benetos, A, Adamopoulos, C, Bureau, JM, Temmar, M, Labat, C, Bean, K, Thomas, F, Pannier, B, Asmar, R, Zureik, M, Safar, M, Guize, L: Determinants of accelerated progression of arterial stiffness in normotensive subjects and in treated hypertensive subjects over a 6-year period. *Circulation,* 105**:** 1202-1207, 2002.
11. DeLong, ER, DeLong, DM, Clarke-Pearson, DL: Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics,* 44**:** 837-845, 1988.
12. Ockene, IS, Matthews, CE, Rifai, N, Ridker, PM, Reed, G, Stanek, E: Variability and classification accuracy of serial high-sensitivity C-reactive protein measurements in healthy adults. *Clin Chem,* 47**:** 444-450, 2001.

Supplemental Data



Supplemental Figure 1. Kaplan-Meier curves for all-cause mortality according to tertiles of baseline (A) serum T_{50}, (B) CPP Fet-A, (C) mono Fet-A, (D) total Fet-A, (E) phosphate, (F) PP_i, (G) CTx, (H) ionized calcium and (I) magnesium concentration. Red, low tertile; blue, intermediate tertile; black, high tertile. Log-rank (Mantel-Cox) *P* values for linear trend are given for each plot. CTx, C-terminal telopeptides; mono Fet-A, monomeric fetuin-A; PP_i, pyrophosphate.

Supplemental Table 1 Crude and multivariable-adjusted hazard ratios for all-cause mortality according to tertiles of baseline CPP Fet-A concentration.

Tertile	Low	Intermediate	High	P^a
		HR (95% CI)	HR (95% CI)	
Crude	Referent	1.60 (0.59 to 4.31)	4.30 (1.77 to 10.5)	0.015
Model 1[b]	Referent	1.55 (0.55 to 4.02)	4.25 (1.64 to 9.76)	0.014
Model 2[c]	Referent	1.24 (0.49 to 2.89)	2.56 (1.38 to 6.70)	0.021
Model 3[d]	Referent	1.25 (0.47 to 2.83)	2.62 (1.00 to 6.74)	0.020
Model 4[e]	Referent	1.18 (0.42 to 2.40)	2.40 (1.00 to 5.05)	0.039
Model 5[f]	Referent	1.07 (0.31 to 1.34)	1.12 (0.40 to 3.04)	0.136

CI, confidence interval; HR, hazard ratio

The lowest tertile was used as the reference group.

[a] *P* for linear trend

[b] model 1 including age and gender

[c] model 2 including covariates from model 1 plus eGFR (CKD-EPI) and proteinuria

[d] model 3 including covariates from model 2 plus phosphate

[e] model 4 including covariate from model 3 plus CVD co-morbidity, SBP, smoking history

[f] model 5 including covariate from model 4 plus hsCRP