Elastin Degradation Is Associated With Progressive Aortic Stiffening and All-Cause Mortality in Predialysis Chronic Kidney Disease

Edward R. Smith, Laurie A. Tomlinson, Martin L. Ford, Lawrence P. McMahon, Chakravarthi Rajkumar, Stephen G. Holt

Abstract—In the large conduit arteries, elastin is important in maintaining vascular compliance. Studies in animal models suggest that elastin degradation may promote arteriosclerotic vascular changes. There is already a well-established link between aortic stiffening and mortality in the general population and in patients undergoing dialysis. Elastin degradation is mediated by several proteases, including matrix metalloproteinase 2 and cathepsin S. Elastin turnover may be inferred by measuring serum levels of elastin-derived peptides. We analyzed the serum concentration of these biomarkers, their endogenous inhibitors, and aortic pulse wave velocity in 200 patients with stages 3 and 4 chronic kidney disease and then serially in a subgroup of 65 patients over 36 months. Serum matrix metalloproteinase 2, cathepsin S, and elastin-derived peptide levels were independently associated with baseline aortic pulse wave velocity and changes in stiffness over the follow-up period. Higher matrix metalloproteinase 2 and elastin-derived peptide levels were also independently associated with preexisting cardiovascular disease. In multivariable Cox regression, higher serum elastin-derived peptide levels were independently associated with increased all-cause mortality (hazard ratio per SD increase=1.78; P=0.021). In predialysis chronic kidney disease, elastin degradation is an important determinant of arterial stiffness and is associated with all-cause mortality. (***Hypertension*****. 2012;59:973-978.)** ● **Online Data Supplement**

Key Words: elastin degradation ■ cathepsin S ■ matrix metalloproteinase 2 ■ aortic stiffness ■ chronic kidney disease

Aging of the central arteries is associated with a number of structural and functional changes as cellular and matrix components become damaged. The fatigue and fracture of elastin fibers are accompanied by an increasingly dysregulated metabolism within the matrix resulting in the accumulation of glycosylated collagen and the deposition of mineral.[1] Loss of elastin leads to an increase in the collagen:elastin ratio and augmented mechanical loading of stiffer collagen fibers. With age, vascular smooth muscle cells (VSMCs) become less contractile, and there are increased rates of cellular senescence and death.[2] Together, these changes contribute to the loss of intrinsic elasticity and progressive stiffening of the arterial wall.[3] In addition to promoting arteriosclerosis, elastin degradation also stimulates intimal VSMC invasion, neointima formation, and plaque destabilization and, thus, may also be an important factor in the pathogenesis of atherosclerotic disease.[4]

Age-associated arterial remodeling is accelerated in patients with chronic kidney disease (CKD), who have a vastly elevated cardiovascular risk compared with the general population.[5] Although not a universal finding, arterial stiffness, as measured by various methods, has been associated with decline in renal function in patients with CKD[6–8] and is a strong predictor of cardiovascular risk and all-cause mortality in patients with end-stage renal failure[9] and in non-CKD populations.[10]

Extracellular matrix turnover is mediated by a number of elastolytic proteinases, including Zn^{2+}/Ca^{2+}-dependent matrix metalloproteinases (MMPs; eg, MMP-2) and cathepsin cysteine proteases, such as cathepsin S. These proteases are mainly secreted by activated macrophage, resident myofibroblasts, or VSMCs but are also expressed intracellularly in a wide range of cell types.[4] The activities of MMP-2 and cathepsin S are partly regulated by the concentration of endogenous inhibitors like tissue inhibitor of metalloproteinases (TIMPs) and cystatin C, respectively.

Recent work in animal models and ex vivo aortic ring cultures has explored the temporal relationship between

Received November 14, 2011; first decision November 30, 2011; revision accepted February 23, 2012.

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The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.111.187807/-/DC1.

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Hypertension **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.111.187807**

matrix degradation and the initiation of vascular calcification.[11–14] As well as leading to a loss of arterial compliance, per se, elastin degradation also appears to be a precursor to mineralization,[15] priming the vessel wall for calcification through the release of elastin peptide fragments (or elastin-derived peptides [EDPs]) that induce osteogenic responses in VSMCs[16] and by providing a nidus for mineral crystal growth. Because there is now strong evidence that elastin degradation may play an important role in vascular disease, we hypothesized that higher circulating cathepsin S, MMP-2, and EDP levels would be associated with increased mortality in our cohort of 200 patients with predialysis CKD.

Methods

A detailed method description is provided in the accompanying online-only Data Supplement.

Study Population

Two-hundred participants were enrolled in a prospective study of cardiovascular risk in patients with stages 3 and 4 CKD. These patients were predominantly attending nephrology outpatient clinics at Brighton and Sussex University Hospitals National Health Service Trust from March 2006 to September 2010. A full history covering renal disease, cardiovascular disease (CVD), and risk factors was obtained at entry to the study.

All of the 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.[17]

For comparison of baseline biochemical data we used random nonfasting serum samples from 152 healthy subjects. Participants gave written informed consent, and the study was approved by local regional ethics committee and conducted in accordance with the Declaration of Helsinki.

Vascular Measurements

All of the vascular measurements were conducted in a quiet, temperature-controlled room. Patients were requested to refrain from smoking or ingesting caffeine before 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, Omron, Tokyo, Japan). The mean of the 2 recordings of systolic blood pressure (SBP) and diastolic blood pressure (DBP) was recorded. Mean arterial pressure (MAP) was determined as follows: MAP=DBP+[(SBP−DBP)/3].

Aortic pulse wave velocity (PWV; APWV) measurement was performed using Complior (Colson, Les Lilas, France). Dedicated mechanotransducers were directly applied to the skin overlying the carotid and femoral arteries, and the distance between the 2 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 published previously.[18] As recommended by recent guidelines,[19] APWV values were adjusted by a scaling factor (×0.8) to allow for comparison with true PWV measurements.

Biochemical Analysis

For nonstandard biochemistries, nonfasting, random clotted blood samples were collected at baseline for study and control groups and at annual follow-up appointments (12, 24, and 36 months) for the study group only, concomitant with vascular assessments. Samples were allowed to clot for 30 minutes, then centrifuged for 10 minutes at 2000 *g* and stored at −80°C until batched analysis. Serum cathepsin S, MMP-2, TIMP-1, TIMP-2, 8 iso-prostaglandin $F_{2\alpha}$, oxidized low-density lipoprotein, and EDP were measured using specific ELISAs. Serum high-sensitivity C-reactive protein (hsCRP) and cystatin C were measured by immunonephelometry.

Statistical Analysis

Continuous variables were expressed as mean±SD or median (25th to 75th percentile), and categorical variables were expressed as proportions (percentages). Multiple linear regression was used to analyze the relationship between baseline markers and APWV, adjusting for previously described determinants of arterial stiffness, including age, MAP, heart rate, estimated glomerular filtration rate (eGFR), and proteinuria.[20]

Mixed linear models, with an unstructured covariance design (random slope and intercept), were used to analyze longitudinal changes in AWPV (dependent variable) over 3 years. Independent variables were baseline age, MAP, eGFR, serum hsCRP, MMP-2, cathepsin S, and EDP concentrations. Maximal likelihood estimations were used to fit the model.

Multiple logistic regression was used to evaluate the relationship between CVD prevalence and candidate marker levels, adjusting for known cardiovascular (age, sex, MAP, smoking history, diabetic status, total cholesterol, triglycerides, hsCRP, and history of preexisting CVD) and renal risk factors (eGFR and proteinuria). Relative risk was expressed as an odds ratio along with 95% CI. Adjusted odds ratios were also calculated to estimate the likelihood of a positive CVD history according to serum MMP-2, cathepsin S, enzyme:inhibitor ratios, and EDP levels categorized by the median value of each.

We analyzed the risk of all-cause death according to serum MMP-2, cathepsin S, enzyme:inhibitor ratios, and natural log-transformed EDP concentration on a continuous scale and after categorization of patients by the median value of each in order account for nonlinear effects. The Kaplan-Meier method was used to estimate unadjusted cumulative survival with log-rank tests for significance. After confirming the proportionality assumption, multivariable Cox hazard models were used to estimate the effect of serum MMP-2, cathepsin S, enzyme:inhibitor ratios, and EDP levels on all-cause mortality. Sequential hierarchal models were used to adjust for confounding factors, first, age and sex, and then for these factors plus renal risk factors eGFR and proteinuria and established cardiovascular risk factors MAP, smoking history (pack years), diabetic status, plasma total cholesterol, triglycerides, serum hsCRP, and history of preexisting CVD. $P<0.05$ was considered significant. All of the analyses were performed with SPSS 19.0.

Results

Cohort Characteristics

Baseline cohort characteristics are summarized in Table S1 (available in the online-only Data Supplement). This is an elderly, predominantly male and hypertensive cohort of 200 CKD patients with a mean eGFR of 33±11 mL/min per 1.73 m^2. Preexisting CVD was prevalent (44%). The mean number of antihypertensive medications taken per patient was 2.1±1.3. Standard mineral parameters were well controlled. Underlying causes of renal dysfunction were hypertension (34.0%), diabetic nephropathy (6.0%), chronic glomerulonephritis (13.5%), vasculitis (5.0%), interstitial nephritis (6.0%), cystic kidney disease (5.5%), obstructive or congenital disease (10.0%), and unknown (20.0%).

Baseline Analysis

Serum MMP-2, cathepsin S, and EDP concentrations were all significantly higher in CKD patients than in healthy controls (Figure S3). Baseline correlates of serum biomarkers and APWV are also shown in the online-only Data Supplement (Table S2). Notably, all 3 markers of elastin turnover were

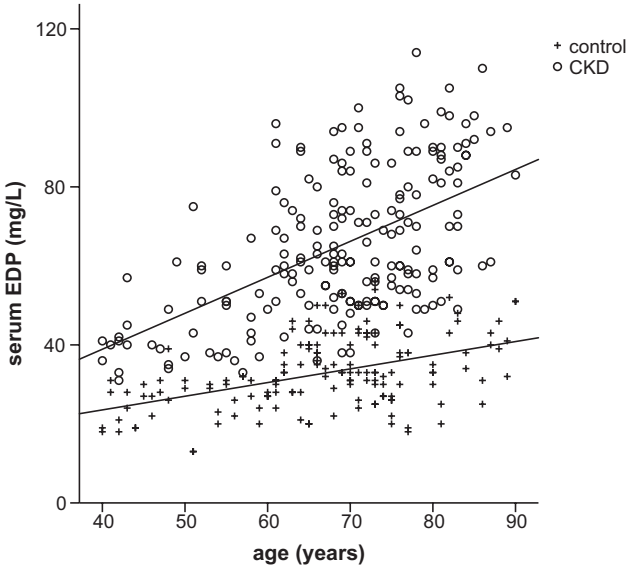

Figure. Linear regression of age-related variation in serum elastin-derived peptides (EDP) concentration in stage 3 and 4 chronic kidney disease (CKD) patients (○: n=200, slope=0.952 mg/L per year; $P<0.001$) and healthy controls (+: n=152, slope=0.347 mg/L per year; $P<0.001$).

associated with increasing age, hsCRP, APWV, lower eGFR, and a history of preexisting cardiovascular comorbidity. Age-related variation in serum EDP concentration is shown in the Figure. Levels in healthy controls showed an increase of ≈0.4 mg/L per year, whereas levels in CKD patients showed an ≈1.0-mg/L per year rise. Age-related variation in MMP-2 and cathepsin S is shown in the Figure S4.

After adjustment for age, MAP, heart rate, eGFR, proteinuria, MMP-2 ($\beta=0.308$; $P<0.001$), cathepsin S ($\beta=0.147$; $P=0.023$), and EDP ($\beta=0.367$; $P<0.001$), all remained significantly associated with APWV (Table S3). eGFR was also associated with APWV in univariate analysis but was not significant in these adjusted models.

Serum MMP-2, cathepsin S, and EDP concentrations were all significantly higher in those with a history for CVD than those without (MMP-2: 538±213 versus 340±129 μg/L, $P<0.001$; cathepsin S: 29.8±7.9 versus 26.6±10.2 μg/L, $P<0.001$; EDP: 62, 50–86 versus 51, 41–64 mg/L, $P=0.001$). After adjustment for known cardiovascular and renal risk factors, higher serum MMP-2 and EDP concentrations, but not cathepsin S, were independently associated with the prevalence of CVD (Table S4). Serum MMP-2:TIMP-1/-2 ratios were significantly associated with CVD history in univariate analysis but lost significance in the multivariable-adjusted model (data not shown). Cathepsin S:cystatin C ratios were not significantly related to CVD history even in unadjusted analysis (data not shown).

Longitudinal Determinants of Aortic Stiffness

APWV and biochemical readings were available in 65 patients at baseline and 12, 24, and 36 months. This subgroup of patients had similar demographic, clinical, and biochemical characteristics to the total study cohort. Levels of serum hsCRP ($P=0.028$) were, however, slightly lower in this subset of patients (Table S5). Figure S5 depicts serial changes in longitudinal parameters. We observed a significant increase in mean APWV (8.3%; $P<0.001$), serum cathepsin S (19.6%; $P=0.001$), MMP-2 (9.3%; $P=0.011$), median serum EDP (9.9%; $P<0.001$), and hsCRP (32.0%; $P<0.001$) and reduction in mean eGFR (6.2%; $P=0.026$) over 3 years (Table S6). No significant change in MAP was recorded ($P=0.122$). Using a mixed linear modeling strategy, we found that, after adjustment for age and MAP, variation in APWV over this time period was significantly associated with changes in serum MMP-2, cathepsin S, EDP, hsCRP, and eGFR (see Table 1).

Table 1. Longitudinal Determinants of Aortic Pulse Wave Velocity in Patients With Stage 3 and 4 Chronic Kidney Disease (n=65)

Parameter	Coefficient (95% CI)	P
Age	0.97 (0.49 to 1.46)	<0.001
MAP	0.005 (−0.014 to 0.009)	0.345
eGFR	−0.21 (−0.47 to 0.05)	0.021
hsCRP	1.25 (0.60–1.90)	<0.001
MMP-2	0.15 (0.08–0.32)	0.009
Cathepsin S	0.09 (0.04–0.22)	0.016
EDP	0.61 (0.38–0.99)	<0.001

EDP indicates elastin-derived peptides; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure; MMP-2, matrix metalloproteinase 2; hsCRP, high-sensitivity C-reactive protein.

Association of Serum MMP-2, Cathepsin S, and EDP Concentration With All-Cause Mortality

During a median follow-up of 1349 days (interquartile range: 683–1717 days), 26 patients (13%) died. Patients were categorized as being above (high) or below (low) the median value of each candidate risk marker. In unadjusted Kaplan-Meier analysis, patients with high MMP-2 and EDP serum levels were at higher all-cause mortality risk than those in the low group (MMP-2: log-rank $P=0.0184$; EDP: log-rank $P=0.0036$). Higher serum MMP-2:TIMP-1/-2 ratios and APWV were also significantly associated with mortality in univariate analysis. No significant difference in mortality was seen when comparing patient outcome by cathepsin S level (log-rank $P=0.148$) or serum cathepsin S:cystatin C ratio (log-rank $P=0.763$; Figure S6).

In univariate Cox regression analysis, cathepsin S was unrelated to all-cause mortality either as a continuous (hazard ratio: 1.21 [95% CI: 0.82–1.79]; $P=0.342$) or categorical variable (hazard ratio: 1.86 [95% CI: 0.79–4.40]; $P=0.155$) or when expressed as a ratio with serum cystatin C concentration (data not shown). MMP-2:TIMP-1/-2 ratios were also not retained in multivariable modeling (data not shown).

MMP-2 was retained in multivariate modeling after adjustment for age, sex, eGFR, proteinuria, MAP, smoking history, diabetic status, cholesterol, triglycerides, and hsCRP (hazard ratio per 1-SD increase in MMP-2: 1.65 [95% CI: 1.72–2.32]; $P=0.004$) but lost significance in predicting outcome after adjustment for preexisting cardiovascular comorbidity (Table S7). Higher serum EDP levels, however, remained significantly associated with all-cause mortality after full multivariable adjustment (HR per 1-SD increase in log-transformed

Table 2. Multivariable Cox Regression Analysis of Serum EDP Concentration All-Cause Mortality in Patients With Stage 3 and 4 Chronic Kidney Disease (n=200)

Parameter	HR	95% CI	*P*
Age (per SD increase)	1.06	1.01–1.12	0.033*
Male sex	1.01	0.57–1.92	0.686
eGFR (per SD increase)	0.83	0.56–1.02	0.053
Proteinuria (per SD increase)	1.22	1.15–1.93	0.038*
Diabetic	1.86	1.07–3.99	0.048*
Cardiovascular comorbidity	3.98	2.24–6.48	0.015*
MAP (per SD increase)	1.01	0.88–1.46	0.374
Smoker	1.15	0.79–2.35	0.074
Cholesterol (per SD increase)	1.02	0.95–1.29	0.158
Triglycerides (per SD increase)	1.13	1.01–1.35	0.047*
hsCRP (per SD increase)	2.33	1.51–4.92	0.021*
APWV (per SD increase)	1.27	0.99–2.98	0.067
EDP (per SD increase)	1.49	1.04–1.98	0.039*

APWV indicates aortic pulse wave velocity; EDP, elastin-derived peptides; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MAP, mean arterial pressure; HR, hazard ratio.

*Data indicate statistically significant result ($P<0.05$).

EDP: 1.78 [95% CI: 1.09–2.90]; $P=0.021$), although the strength of this relationship was significantly attenuated by the addition of APWV into the model (Table 2). A higher APWV was also associated with mortality risk after adjustment for age ($P=0.004$), but did not achieve significance in the fully adjusted model.

Discussion

This is the first study to examine the relationship between serum markers of elastin degradation, arterial stiffness, and mortality in the setting of predialysis CKD. Overall, the data presented here support the hypothesis that dysregulated elastin metabolism may be an important factor in the accelerated vascular disease seen in patients with CKD. In this study, serum MMP-2, cathepsin S, and EDP levels were higher in CKD patients compared with healthy controls of a similar age, and levels increased at a higher rate than in non-CKD subjects. Higher MMP-2 and EDP levels were strongly associated with the presence of preexisting CVD, independent of established cardiovascular and renal risk factors. We also found a strong independent association among MMP-2, cathepsin S, EDP, and APWV at baseline and a significant temporal relationship between changes in these markers and aortic stiffening over 3 years. Finally, we found that higher serum EDP concentrations were independently associated with increased all-cause mortality.

With respect to MMP-2, our data are consistent with previous reports showing that circulating MMP-2 levels are elevated in predialysis CKD[21] and are higher in those with cardiovascular comorbidity.[21,22] MMP-2 levels have also previously been strongly associated with APWV in a hypertensive but otherwise healthy cohort.[23] Similarly, existing data on EDPs indicate a strong correlation with age and history of CVD.[24]

Although the weight of evidence favors cathepsin S being involved in atherosclerotic vascular disease, the landmark article by Aikawa et al[12] showed that ablation of cathepsin S in uremic rats halted the development of arterial medial calcification, thus suggesting a potential role in arteriosclerosis. Given that vascular mineralization is a strong determinant of large vessel stiffness, it seemed logical to investigate whether higher serum cathepsin S levels were associated with increased APWV in our cohort. Although serum cathepsin S levels were not independently associated with CVD or mortality in this study, findings somewhat inconsistent with previous reports,[25,26] concentrations were, however, independently associated with APWV at baseline and over 3 years. Clearly, therefore, although the role of cathepsin S in the pathogenesis of vascular disease in human CKD requires further investigation, our novel data are consistent with it being an important factor in the progression of arteriosclerosis.

Unlike EDP levels, neither serum MMP-2 nor cathepsin S were independently associated with all-cause mortality. Because elastinolytic activity may be better described by the balance between enzyme and inhibitor levels, we also tested whether MMP-2:TIMP-1, MMP-2:TIMP-2, and cathepsin S:cystatin C ratios were associated with arterial stiffness and outcome. In all of the analyses, enzyme concentrations alone were more strongly related with other biochemical variables (eg, hsCRP), APWV, and outcome measures. We, therefore, question the use of these ratios. Critically, it is not clear whether serum concentrations reflect tissue levels of these inhibitors. For instance, levels of serum cystatin C, the most abundant endogenous inhibitor of cathepsin S, are increased in those with atherosclerotic disease, yet levels within the vessel wall appear reduced.[27] However, it should be noted that serum TIMP-1 levels are subject to significant diurnal variation, whereas TIMP-2, on the other hand, might be subject to exercise-related variation.[28] Consequently, we acknowledge that random measurements, as reported here, may not provide the most accurate assessment of inhibitor levels.

The lack of significant association between higher MMP-2 concentrations and mortality after adjustment for cardiovascular comorbidity is perhaps unsurprising given that elevated MMP-2 levels are themselves so strongly predictive of a positive CVD history in this cohort. Almost 50% of this elderly cohort has significant preexisting CVD. Although this may in part reflect a lack of power to detect a significant independent association between MMP-2 (or cathepsin S) and mortality, it is nevertheless important to underline that, after the same multivariable adjustment, EDP concentration remained significantly associated with outcome. Serum EDP is an attractive candidate vascular risk marker, because its circulating levels reflect the balance of elastin-degrading enzyme activity and tissue inhibitor levels and, therefore, theoretically provides a better measure of elastin turnover than the concentration or activity of a single elastinolytic enzyme.

It is possible that elastin degradation, as indicated by elevated serum levels of EDP, may relate to increased mortality risk through a number of pathways. First, it may

relate via a direct effect on central artery compliance through loss of elastin fibers leading to increased stiffness and downstream effects on the heart. Second, elastin degradation may prime the vessel for mineralization (also reducing compliance) by nucleating mineral deposition and inducing the osteogenic differentiation of VSMCs. Such a temporal relationship is now convincingly supported by animal models that demonstrate that elastin fragmentation can lead to vascular calcification in CKD. Thinning of the internal elastic lamina colocalizes with mineral deposition, MMP-2 activation, and Runx2 upregulation in neighboring VSMCs.[8,10] These studies also show that MMP-2 inhibition or cathepsin S ablation in the context of uremia abrogates the development of arterial medial calcification.[8,9] Interestingly, calcified aortic elastin is more susceptible to proteolysis than uncalcified elastin,[29] raising the possibility of a vicious cycle in which elastin fragmentation leads to mineralization, which, in turn, promotes further degradation. Unfortunately, we do not have imaging data to directly examine the relationship between elastin degradation markers and aortic calcification burden in our patients, and future work should certainly address this point.

Intriguingly, EDPs may themselves be directly pathogenic. Engagement of the elastin-laminin receptor by EDPs has been shown to elaborate osteogenic responses in VSMCs, potentially perpetuating arterial mineralization.[16,30] Although the study by Hosaka et al[30] showed that the action of EDPs was to enhance the effect of phosphate on VSMC osteoblastic differentiation and mineralization, the role of phosphate in this context is uncertain. The original study by Simionescu et al[16] showed that EDPs induced upregulated expression of MMP-2, Runx2, osteocalcin, and alkaline phosphatase in rat aortic VSMCs, in normophosphatemic media, and in the absence of other mineralizing agents. Hosaka et al[30] also used phosphate concentrations substantially in excess of those seen in the extracellular fluid of patients with CKD 3 and 4, and they are, therefore, not entirely representative of our patients. Although phosphate is considered to be a key driver of vascular mineralization (and, hence, stiffness) in CKD, we found no significant relationship between plasma phosphate levels and APWV ($P=0.854$) or mortality (log-rank $P=0.669$) in this cohort. Phosphate was also not significantly correlated with MMP-2, cathepsin S, or EDPs. On the contrary, EDP levels were strongly correlated with plasma calcium concentration, an important activator of MMPs and regulator VSMC-derived matrix vesicle mineralization.[31]

Chronic exposure of the elastin-laminin receptor to saturating levels of EDPs also appears to have effects on the endothelium and cells of the innate immune system. In endothelial cells, there is evidence that elastin-laminin receptor signaling results in increased free radical production and decreased NO generation.[32] The latter may well impact stiffness through impairment of NO-mediated vasodilatatory pathways. EDPs exert potent chemotactic effects on monocytes and neutrophils, attracting them to sites of elastin degradation[33] and stimulating them to produce free radicals, chemokines, cytokines, and proteases (including MMPs),[34] further perpetuating the cycle of inflammation and arterial extracellular matrix remodeling.[35]

Finally, independent of effects on arterial stiffness, as already noted, elastin degradation may promote intimal invasion of VSMCs, atherosclerotic plaque destabilization[36] and may predispose to aneurysmal change.[37] Hence, elastin degradation may impact on cardiovascular mortality through arteriosclerotic and atherosclerotic processes.

Although APWV does not appear to be an independent determinant of mortality in this cohort, the fact that the strength of the association between serum EDP and mortality is significantly attenuated by the addition of APWV to the multivariable model suggests a role for arterial stiffening in mediating this increased risk. Similarly, the inclusion of hsCRP in the model also significantly moderates the risk associated with higher EDP levels and, in our view, attests to the importance of inflammatory processes as key drivers for vascular disease in CKD patients. Importantly, we have been able to demonstrate a strong longitudinal relationship among MMP-2, cathepsin S, and EDP levels with systemic inflammation (hsCRP). Speculatively, chronic stimulation of the immune system and its downstream effects on vascular cells seen in patients with CKD may represent an exaggerated form of the perturbations seen during aging.[38]

We readily acknowledge that this study is limited by its size (n=200) and event rate (13%). This may account for the lack of independent association between APWV and mortality. For the same reason we were unable to look specifically at cardiovascular mortality as an outcome variable; therefore, although we assume the association with all-cause mortality to be mediated by increased cardiovascular risk, this remains unproven. This epidemiological study can only provide limited mechanistic insight into the pathogenesis of vascular disease in these patients, and further work is needed to carefully dissect out the precise mechanisms by which elastin turnover may contribute to mortality risk.

Here we have shown that serum levels of EDPs are elevated in patients with CKD and increase with age at a higher rate than in healthy controls. These products are independently associated with progressive aortic stiffening and relate to changes in inflammatory status and elastin-degrading enzyme concentration. Uniquely, we show that higher serum EDP concentrations are a novel independent risk marker for all-cause mortality in CKD.

Perspectives

Patients with CKD present a paradigm of accelerated vascular aging. This is evident at the histological level by changes in the extracellular matrix, including loss of elastin and mineral deposition, as well as changes in cellular components in large arteries. These changes result in aortic stiffening and loss of function of the major vessels. Changes in VSMCs appear to be central to the pathogenesis of these changes. The drivers to the phenotypic changes that occur with renal failure are the subject of intense scrutiny, because modulating them may be a key step in abrogating this process. We already know that inflammation, oxidative stress, and mineral imbalance are important drivers to this process, but this study sheds further light on how these might link with the pathology.

Sources of Funding

We gratefully acknowledge the support from the Sussex Kidney Unit and the Clinical Investigation and Research Unit, Brighton and Sussex University Hospitals, United Kingdom, and the Department of Renal Medicine, Eastern Health Clinical School, Monash University, Australia.

Disclosures

None.

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Hypertension
Journal of the American Heart Association

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Hypertension. 2012;59:973-978; originally published online March 12, 2012;
doi: 10.1161/HYPERTENSIONAHA.111.187807
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563

ONLINE SUPPLEMENT

ELASTIN DEGRADATION IS ASSOCIATED WITH PROGRESSIVE AORTIC STIFFENING AND ALL-CAUSE MORTALITY IN PRE-DIALYSIS CKD

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Original Scientific Contribution

Running Title: elastin degradation in pre-dialysis CKD

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Supplemental Methods

Study Population

200 participants were enrolled in a prospective study of cardiovascular risk in patients with stage 3 & 4 CKD. These patients were predominantly attending nephrology outpatient clinics at Brighton and Sussex University Hospitals NHS Trust from March 2006 to September 2010. 96.5% were Caucasian, 2% Arab, 1% South Asian, 0.5% Black African. A full history covering renal disease, cardiovascular disease and risk factors was obtained 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[1].

For comparison of baseline biochemical data we used random non-fasting serum samples from 152 healthy subjects. These individuals were recruited from the same local healthcare authority and had no history of cardiovascular disease (exclusion criteria: previous myocardial infarction, stroke, heart failure, or receiving lipid-lowering/antihypertensive therapy), type 2 diabetes mellitus, malignancy, recent infection or trauma, with a normal glomerular filtration rate (eGFR > 60 mL/min/1.73m^2) and a urinary total protein:creatinine ratio (<10 mg/mmol). Relevant medical history was self-reported by the participants or accessed from records held by their General Practitioner.

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

Vascular measurements

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: MAP = 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[2]. As recommended by recent guidelines[3], carotid-femoral PWV values were adjusted by a scaling factor (x 0.8) to allow for comparison with studies using 'real' PWV distance measurements. At baseline, APWV measurements were available for 185 subjects,

whilst 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). APWV measurements were available in 65 patients at 12, 24, and 36 months after the baseline visit.

Routine Biochemical analysis

Standard biochemical analysis was performed using a routine automated analyser (Roche Modular, Haywards Heath, UK). Estimated glomerular filtration rate (eGFR) was calculated using the 4-variable equation derived from the Modification of Diet in Renal Disease (MDRD) study[4]. Serum 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 PTH (iPTH) was measured using Elecsys reagents for the Modular Analytics E170 immunoanalyser (Roche Diagnostics, Burgess Hill, UK). Random plain urine was collected for determination of proteinuria and albuminuria at baseline.

Serum ELISA measurements

For non-standard biochemistries, non-fasting, random clotted blood samples were collected at baseline for study and control groups and at annual follow-up appointments (12, 24 and 36 months) for the study group only, concomitant with vascular assessments. Samples were allowed to clot for 30 minutes, then centrifuged for 10 min at 2000 *g* and stored at -80°C until batched analysis.

Unless otherwise stated, all reagents and chemicals were analytical grade and obtained from Sigma (Sigma-Aldrich, Dorset, UK). Serum total cathepsin S concentration was measured using a commercially available DuoSet ELISA development kit (R&D systems, Abingdon, UK) that detects pro-, mature and cystatin-complexed forms and is calibrated using an NS0-expressed recombinant human cathepsin S standard. This assay shows no cross-reactivity with cathepsin L. In-house validation of this assay gave a mean recovery of 94.5 ± 8.8 % at 15 µg/L using spiked serum samples, and within- and between-batch imprecision at 13.5 µg/L were 6.4% and 7.1% respectively. The limit of detection was 0.15 µg/L. Serum elastin-derived peptide (EDP) concentration was determined using a competitive ELISA based on the method described by Sivaprasad *et al*[5]. In our hands, the functional sensitivity of this assay was 0.4 µg/L (concentation at which CV <20%) and within- and between-batch imprecision at 50 mg/L were 7.4 % and 7.5 % respectively. Both assays were performed on a fully-automated Triturus ELISA workstation (Grifols, Cambridge, UK). Details of these assays are provided as Supplemental Material. Serum MMP-2 concentration was measured using a human quantikine ELISA kit (#DMP2F0, R&D Systems, Abingdon, UK) according to the manufacturer's instructions. This assay does not detect TIMP-bound MMP-2. Within- and between-batch imprecision at 300 µg/L were 6.2 % and 6.8 % respectively. oxLDL was measured by commercially available ELISA (Mercodia, Uppsala, Sweden). Limit of detection was 6.0 U/L and between-batch imprecision was 4.3% at 40 U/L. Serum 8 iso-prostaglandin $F_{2\alpha}$ ($8iPGF_{2\alpha}$) was measured using a commercial ELISA kit (Cayman Chemical, Ann Arbor, Michigan, USA). Limit of detection was 2.7 ng/L and between-batch imprecision was 10.1% at 300 ng/L. Serum TIMP-1 & -2 were measured using kits from R&D Systems. Between-batch imprecision was <6.5%.

Determination of serum EDP concentration

Serum elastin-derived peptide (EDP) concentration was determined using a competitive ELISA based on the method described by Sivaprasad *et al*[5]. Briefly, wells of high-binding clear polystyrene 96-well EIA plates (Costar #2529, R&D systems, Abingdon, UK) were coated with 150 µL (1.5 mg/L) soluble human aortic elastin peptides (#RY53, Elastin Products Co., Missouri, USA) in 0.1 sodium carbonate buffer (pH 9.0) for 12 h at 4°C. Plates were then washed five times with 300 µL PBS (pH 7.3) containing 0.05% (vol/vol) Tween 20 (PBST). The assay was calibrated using dilutions of human aortic elastin peptides (10-150 µg/L) in PBST containing 8% (wt/vol) bovine serum albumin (Fraction V, Protease free BSA #82-045, Millipore, Watford, UK) (PBST- 8% BSA). These dilutions were pre-incubated with rabbit anti-human aortic elastin polyclonal antisera (1:1000) in PBST-8% BSA for 12 h at 4°C. 100 µL of this mixture was then transferred to the pre-coated plates and incubated for 1 h at 23°C. After washing the plate 5 times (300 µL/well) with PBST, 150 µL HRP-conjugated goat anti-rabbit polyclonal IgG antibody (#sc-2007, Santa Cruz Biotechnology, California, USA) was added to each well and then incubated for a further 1 h at 23°C (1:2000 dilution in PBST-8% BSA). After further washing (300 µL/well PBST, 5 times), 100 µL of ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) in 0.05 M phosphate-citrate buffer (pH 5.0) was added to each well and incubated for 1 h at 23°C before measuring the absorbance at 495 nm. Standards and samples were analysed in triplicate. A typical standard curve is given in Figure S1.

Determination of serum cathepsin S concentration

Capture and detection antibody, streptavidin-HRP conjugate, and standards were obtained from R&D Systems as an ELISA DuoSet development kit (Abingdon, UK). EIA plates were prepared as follows. Wells of high-binding clear polystyrene 96-well plates (Costar #2529, R&D systems, Abingdon, UK) were coated 100 µL goat anti-human cathepsin S antibody in PBS (0.8 mg/L) for 12 h at 4°C. The plate was then washed three times with wash buffer (300 µL PBST) before blocking the plates with 300 µL PBST-1% BSA) for 1h at 23°C. The plates were then washed a further time as above before assay. 100 µL of diluted sample (1:100) or standard was added to each appropriate well and then incubated at 23°C for 2 h. The plate was then washed three times using PBST before the addition of 100 µL biotinylated goat anti-human cathepsin S detection antibody (400 µg/L in PBST-1% BSA) and incubation for a further 2 h at 23°C. After another wash cycle, 100 µL of HRP-streptavidin conjugate (lot-sepcific, diluted according to the manufacturers' instructions) was added to each well and then incubated in the dark for 20 min at 23°C. After a further wash cycle, 100 µL TMB/H_2O_2 substrate solution was added to each well and incubated in the dark for 20 min at 23°C. 50 µL stop solution (2 M H_2SO_4) and then the absorbance was determined at 450 nm. A seven-point standard curve (4-PL fit) was generated using readings of two-fold serial dilutions of recombinant human cathepsin S standard in PBST-1% BSA. A typical standard curve for this assay is given in Figure S2.

Assessment of analytical characteristics

For the assessment of analytical performance, we defined the limit of detection as the concentration of analyte (measured 20 times in a single batch) that generates a signal 3 standard deviations above the mean for an analyte-free matrix. Imprecision was evaluated by measuring 10 replicates of pooled plasma samples at 3 different concentrations in five independent batches. To assess recovery, we spiked 10 individual pre-diluted plasma samples (encompassing a range of endogenous concentrations) with low, intermediate and high

concentrations of either human aortic elastin peptides (Elastin Products Co., Missouri, USA) or recombinant human cathepsin S (R&D Systems, Abingdon, UK.

Definition of pre-existing cardiovascular disease

Pre-existing cardiovascular co-morbidity was defined as a history of transient ischaemic attack, stroke, myocardial infarction, angina or if the patient had undergone treatment for cardiovascular disease (e.g. coronary artery bypass grafting or angioplasty).

Definition of primary endpoint

Survival data was gathered prospectively during the study. Data censoring was performed on 1/8/11. The survival status of patients was then confirmed using electronic hospital computer records.

Statistical analysis

Continuous variables were expressed as mean ± SD or median (25^{th}-75^{th} percentile) and compared by *t*-test or Mann Whitney-U test as appropriate. Skewed variables were natural log-transformed. Categorical variables were expressed as proportions (%) and compared using the chi-squared test. Multiple linear regression was used to analyse the relationship between baseline markers and APWV, adjusting for previously described determinants of arterial stiffness[6], namely: age, MAP, heart rate, eGFR and proteinuria.

Mixed linear models with an unstructured covariance structure (random slope and intercept) were used to analyse longitudinal changes in AWPV (dependent variable) over 3 years. Independent variables were baseline age, MAP, eGFR, serum hsCRP, MMP-2, cathepsin S and EDP concentrations. Maximal likelihood estimations were used to fit the model.

Multiple logistic regression was used to evaluate the relationship between CVD prevalence and candidate marker levels, adjusting for covariates significantly associated with CVD prevalence in univariate analysis and known cardiovascular (age, gender, MAP, smoking history, diabetic status, total cholesterol, triglycerides, hsCRP, and history of pre-existing CVD) and renal risk factors (eGFR, proteinuria). Relative risk was expressed as an Odds Ratio (OR) along with 95% CI. Adjusted OR were also calculated to estimate the likelihood of a positive CVD history according to serum MMP-2, cathepsin S, enzyme-to-inhibitor ratios and EDP levels categorised by the median value of each.

We analysed the risk of all-cause death according to serum MMP-2, cathepsin S, enzyme-to-inhibitor ratios and natural log-transformed EDP concentration on a continuous scale and after categorisation of patients by the median value of each in order account for nonlinear effects. The Kaplan-Meier method was used to estimate unadjusted cumulative survival with log-rank tests for significance. After confirming the proportionality assumption, multivariable Cox hazard models were used to estimate the effect of serum MMP-2, cathepsin S, ezyme-to-inhibitor ratios and EDP levels on all-cause mortality. Sequential hierarchal models were used to adjust for confounding factors, firstly age and gender and then for these factors plus renal risk factors: eGFR, proteinuria and established cardiovascular risk factors: MAP, smoking history (pack years), diabetic status, plasma total cholesterol, triglycerides, serum hsCRP, and history of pre-existing CVD. Sensitivity analyses suggested that the choice of blood pressure parameter (SBP vs. MAP) had no significant impact on the modelling in logistic or Cox regression. Similarly addition of both parameters did not significantly

improve the fit of the model. We have chosen to use MAP as this most closely represents the distending pressure of the artery at the time of measurement.

$P < 0.05$ was considered significant. All analyses were performed with SPSS 19.0

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Supplemental Results

Table S1 Baseline characteristics of CKD and control group (n=200)

Characteristic	CKD	Controls
Age (yr)	69 ± 11	68 ± 12
Gender (% male)	72	68
Cardiovascular comorbidity (%)	44	-
History of diabetes (%)	26	-
Alcohol intake (units/wk)	7.5 ± 9.5	NA
Smoking (packs/yr)	17.6 ± 26.6	NA
BMI (kg/m^2)	28.4 (25.4-33.6)	NA
Systolic BP (mmHg)	151 ± 22	NA
Diastolic BP (mmHg)	81 ± 11	NA
Mean arterial pressure (mmHg)	105 ± 13	NA
Pulse pressure (mmHg)	70 ± 19	NA
Heart rate (bpm)	70 ± 12	NA
APWV (m/s)*	10.4 ± 2.1	NA
Laboratory measurements		
eGFR ($mL/min/1.73m^2$)	33 ± 11	>60
Proteinuria (mg/mmol)	27.7 (13.3-69.0)	<10
Albuminuria (mg/mmol)	5.4 (1.8-32.4)	<2.5
Plasma albumin (g/L)	43 ± 3	NA
Plasma adjusted calcium (mmol/L)	2.29 ± 0.11	NA
Plasma phosphate (mmol/L)	1.08 ± 0.20	NA
Plasma iPTH (ng/L)	75 (50-118)	NA
Haemoglobin (g/dL)	12.7 ± 1.7	NA
Serum hs-CRP (mg/L)	2.30 (0.95-5.76)	NA
Plasma total cholesterol (mmol/L)	4.2 ± 1.2	NA
Plasma HDL-cholesterol (mmol/L)	1.19 ± 0.41	NA

Plasma triglycerides (mmol/L)	1.48 ± 0.45	NA
Serum oxLDL (U/L)	67 (46-130)	NA
Serum 8 iso-prostaglandin $F_{2\alpha}$ (ng/L)	301 ± 128	NA
Serum MMP-2 (µg/L)	421 ± 18.5	337 ± 147
Serum TIMP-1 (µg/L)	102 ± 55	NA
Serum TIMP-2 (µg/L)	1.22 ± 0.66	NA
Serum MMP-2/TIMP-1 ratio	5.6 ± 4.8	NA
Serum MMP-2/TIMP-2 ratio	465 ± 374	NA
Serum cathepsin S (µg/L)	27.0 ± 0.81	18.2 ± 6.6
Serum cystatin C (mg/L)	1.87 ± 0.05	NA
Serum cathepsin S/ cystatin C ratio (µg/mg)	15.4 ± 0.55	NA
Serum EDP (mg/L)	57 (43-78)	36 (30-45)
Medication use		
ACEi/ARB (%)	65	-
CCB (%)	46	-
Diuretic (%)	55	-
Beta blocker (%)	33	-
Statin (%)	60	-
Ca-based phosphate binder or Vit D (%)	14	-
Bisphosphonate (%)	8	-

Data are mean ± SD or median (25^{th}-75^{th} percentile)

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; APWV, aortic pulse wave velocity; BMI, body mass index; BP, blood pressure; CCB, calcium channel blocker; EDP, elastin derived peptide; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MMP-2, matrix metalloproteinase-2; iPTH, intact parathyroid parathyroid hormone; oxLDL, oxidised low density lipoprotein; TIMP, tissue inhibitor of metalloproteinase; Vit D, vitamin D

*adjusted for scaling factor x 0.8

NA – not assessed

Table S2. Univariate associations between baseline serum MMP-2, cathepsin S, EDP, APWV and other characteristics in 200 patients with stage 3 & 4 CKD

Characteristic	MMP-2	Cathepsin S	EDP	APWV
Age	0.160*	0.207*	0.366‡	0.536‡
Gender	0.026	-0.086	0.044	-0.008
Cardiovascular comorbidity	0.500‡	0.171*	0.233†	0.343‡
History of diabetes	0.272‡	0.245‡	0.056	0.145*
Alcohol intake	-0.050	-0.005	-0.014	-0.057
Smoking	0.385‡	0.017	0.180*	0.148*
BMI§	0.149*	0.083	0.009	0.009
Systolic BP	0.117	0.050	0.096	0.267‡
Diastolic BP	-0.012	-0.143	-0.133	-0.183*
Mean arterial pressure	0.054	0.089	0.014	0.068
Pulse pressure	0.141*	0.148*	0.183*	0.423‡
Heart rate	-0.079	-0.108	0.090	0.062
APWV	0.456‡	0.251†	0.547‡	-
eGFR	-0.159*	-0.229†	-0.349‡	0.152*
Proteinuria§	0.085	0.019	0.179*	0.059
Albuminuria§	0.093	-0.012	0.162*	0.046
Plasma albumin	-0.152*	0.013	-0.141*	0.116
Plasma adjusted calcium	0.065	0.058	0.188†	0.037
Plasma phosphate	0.122	0.073	0.041	0.016
Plasma iPTH§	0.138	0.122	0.146*	0.142
Haemoglobin	-0.102	-0.101	-0.147*	-0.177*
Serum hs-CRP§	0.304‡	0.178†	0.391‡	0.238†
Plasma total cholesterol	-0.020	-0.077	-0.099	-0.126
Plasma HDL-cholesterol	-0.069	-0.017	-0.056	-0.069
Plasma triglycerides	0.166*	-0.017	0.049	0.041
Serum oxLDL	0.555‡	0.004	0.059	0.044
Serum 8 iso-prostaglandin $F_{2\alpha}$	0.312†	0.055	0.112	-0.036
ACEi/ARB use	-0.102	0.030	-0.107	-0.239†
CCB use	0.102	0.072	0.027	0.134
Diuretic use	0.056	0.107	0.014	0.016
Beta blocker use	0.112	0.107	0.001	0.058
Statin use	-0.006	-0.092	-0.110	0.053
Ca-based phosphate binder/Vit D use	-0.059	0.004	-0.025	0.050
Bisphosphonate use	-0.062	0.005	-0.086	-0.023
Serum MMP-2	-	0.151*	0.283‡	0.402‡
Serum TIMP-1	-0.043	0.044	-0.030	-0.029
Serum TIMP-2	0.143*	0.044	-0.029	-0.042
MMP-2/TIMP-1 ratio	0.624‡	0.120	0.167*	0.245†
MMP-2/TIMP-2 ratio	0.594‡	0.120	0.179*	0.215†
Serum cathepsin S	0.151*	-	0.270‡	0.249†
Serum cystatin C	0.237†	0.207*	0.320‡	0.216*
Cathepsin S/cystatin C ratio	-0.081	0.670‡	0.047	0.070
Serum EDP§	0.283‡	0.270‡	-	0.550‡

*P<0.05, †P<0.01, ‡P<0.001.

§natural log-transformed

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; APWV, aortic pulse wave velocity; BMI, body mass index; BP, blood pressure; CCB, calcium channel blocker; EDP, elastin derived peptide; eGFR, estimated glomerular filtration

rate; hs-CRP, high-sensitivity C-reactive protein; MMP-2, matrix metalloproteinase-2; iPTH, intact parathyroid parathyroid hormone; oxLDL, oxidised low density lipoprotein; TIMP, tissue inhibitor of metalloproteinase; Vit D, vitamin D

Table S3. Multiple linear regression analysis of determinants of baseline APWV in 185 patients with stage 3 & 4 CKD

Parameter	β	P value
Age	0.50	<0.001
MAP	0.04	0.567
Heart rate	0.07	0.195
eGFR	0.02	0.740
Proteinuria*	0.12	0.066
MMP-2	**0.31**	**<0.001**
model R^2 = 0.41		

Parameter	β	P value
Age	0.53	<0.001
MAP	0.05	0.477
Heart rate	0.06	0.359
eGFR	0.01	0.866
Proteinuria*	0.16	0.021
Cathepsin S	**0.15**	**0.023**
model R^2 = 0.33		

Parameter	β	P value
Age	0.43	<0.001
MAP	0.03	0.599
Heart rate	0.02	0.767
eGFR	0.05	0.423
Proteinuria*	0.10	0.124
EDP	**0.37**	**<0.001**
model R^2 = 0.43		

*natural log-transformed

Table S4. Odds ratios (OR) of CVD according to (A) MMP-2, (B) cathepsin S and (C) EDP concentration (per SD increase or categorized by median) in patients with stage 3 & 4 CKD (n=200)

A

Model	Per 1SD increase in MMP-2			MMP-2 ≤378 vs. >378 µg/L		
	OR	95% CI	*P*	OR	95% CI	*P*
Crude	1.01	1.01 to 1.02	0.001*	4.23	2.33 to 7.68	0.001*
Multivariable adjusted	3.11	1.94 to 5.01	0.001*	2.41	1.81 to 4.91	0.016*

B

Model	Per 1SD increase in cathepsin S			cathepsin S ≤26.7 vs. >26.7 µg/L		
	OR	95% CI	*P*	OR	95% CI	*P*
Crude	1.04	1.00 to 1.07	0.018*	1.77	1.01 to 3.12	0.047*
Multivariable adjusted	1.28	0.91 to 1.79	0.153	1.32	0.86 to 1.62	0.123

C

Model	Per 1SD increase in log EDP			EDP ≤56 vs. >56 mg/L		
	OR	95% CI	*P*	OR	95% CI	*P*
Crude	4.23	1.74 to 10.3	0.002*	2.51	1.41 to 4.46	0.002*
Multivariable adjusted	1.98	1.11 to 2.97	0.018*	2.01	1.06 to 4.12	0.029*

*indicates statistically significant result (P<0.05)

Table S5. Characteristics of follow-up group with serial measurements (n=65)

Characteristic	Value
Age (yr)	67.8 ± 11.8
Gender (% male)	72
Cardiovascular comorbidity (%)	36
History of diabetes (%)	21
BMI (kg/m^2)	28.7 (25.6-32.3)
Systolic BP (mmHg)	152 ± 20
Mean arterial pressure (mmHg)	106 ± 12
Pulse pressure (mmHg)	70 ± 17
Heart rate (bpm)	70 ± 12
APWV (m/s)*	9.6 ± 1.7
No. of anti-hypertensive's	2.2 ± 1.3
Laboratory measurements	
eGFR (mL/min/1.73m^2)	35.2 ± 11.3
Albuminuria (mg/mmol)	3.9 (1.4-16.5)
Serum hs-CRP (mg/L)	1.75 (0.68-3.55)†

Data are mean ± SD or median (25th-75th percentile)

APWV, aortic pulse wave velocity; BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein

*scaling factor (x0.8) applied to APWV results

†indicates significant ($P<0.05$) difference from total CKD cohort characteristics (see Supplemental Table S1)

Table S6. Serial changes in mean MAP, eGFR, APWV, serum MMP-2, cathepsin S and EDP concentration over 36 months in 65 patients with stage 3 & 4 CKD

Variable	Time (months)				P for trend
	0	12	24	36	
MAP (mmHg)	100 ± 12	101 ± 13	98 ± 11	99 ± 11	0.122
eGFR (mL/min/1.73m^2)	34.7 ± 11.2	33.9 ± 12.0	33.0 ± 13.1	32.6 ± 13.6	0.026
APWV (m/s)*	9.6 ± 2.0	9.9 ± 2.0	10.3 ± 2.0	10.6 ± 2.2	<0.001
MMP-2 (μg/L)	369 ± 215	373 ± 246	382 ± 205	386 ± 205	0.011
Cathepsin S (μg /L)	28.9 ± 10.4	29.8 ± 14.0	30.1 ± 12.2	31.4 ± 17.9	0.001
EDP (mg/L)	51 ± 22	53 ± 27	57 ± 26	59 ± 24	<0.001
hsCRP (mg/L)	1.78 ± 3.0	2.05 ± 3.0	1.98 ± 4.0	2.22 ± 4.0	<0.001

Data are mean ± SD

*adjusted for scaling factor x 0.8

APWV, aortic pulse wave velocity; EDP, elastin-derived peptide; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MMP-2, matrix metalloproteinase-2; MAP, mean arterial pressure

Table S7 Multivariable Cox regression analysis of serum MMP-2 concentration and all-cause mortality in patients with stage 3 & 4 CKD (n=200)

Parameter	HR	95% CI	P
Age (per SD increase)	1.12	1.00 to 1.24	0.030*
Male Gender	1.06	0.68 to 2.01	0.600
eGFR (per SD increase)	0.83	0.63 to 1.01	0.055
Proteinuria (per SD increase)	1.49	1.06 to 1.71	0.041*
Diabetic	2.17	1.08 to 3.99	0.044*
Cardiovascular comorbidity	4.57	2.52 to 5.89	0.009*
MAP (per SD increase)	1.00	0.98 to 1.26	0.322
Smoker	1.13	0.93 to 1.82	0.063
Cholesterol (per SD increase)	1.01	0.90 to 1.40	0.162
Triglycerides (per SD increase)	1.14	1.00 to 1.55	0.041*
hsCRP (per SD increase)	2.58	1.56 to 4.63	0.022*
MMP-2 (per SD increase)	1.25	0.84 to 1.86	0.265

*indicates statistically significant result ($P<0.05$)

eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MAP, mean arterial pressure; MMP-2, matrix metalloproteinase-2

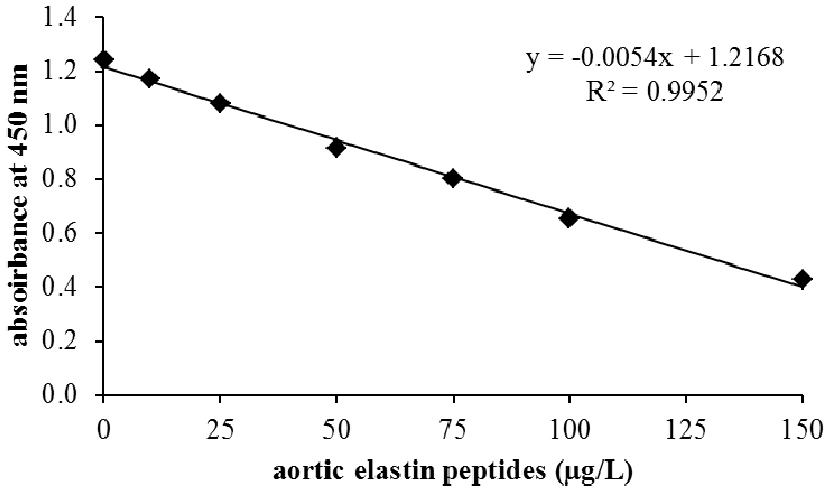

Figure S1. Representative standard curve for serum EDP.

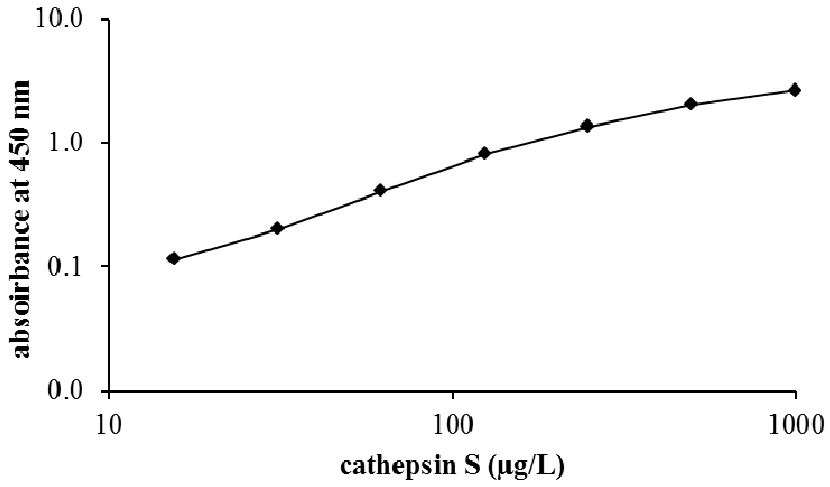

Figure S2. Representative standard curve for serum cathepsin S.

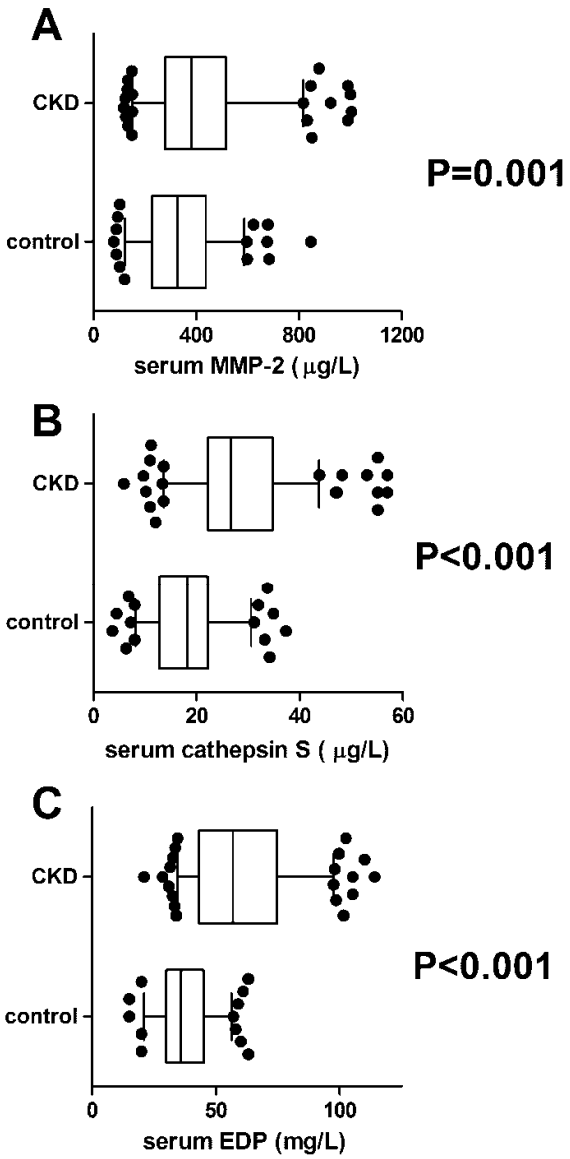

Figure S3 Distribution of serum (A) MMP-2, (B) cathepsin S and (C) EDP concentrations in patients with stage 3 & 4 CKD (n=200) and in healthy controls (n=152). All three serum biomarkers were significantly higher in CKD cohort than controls.

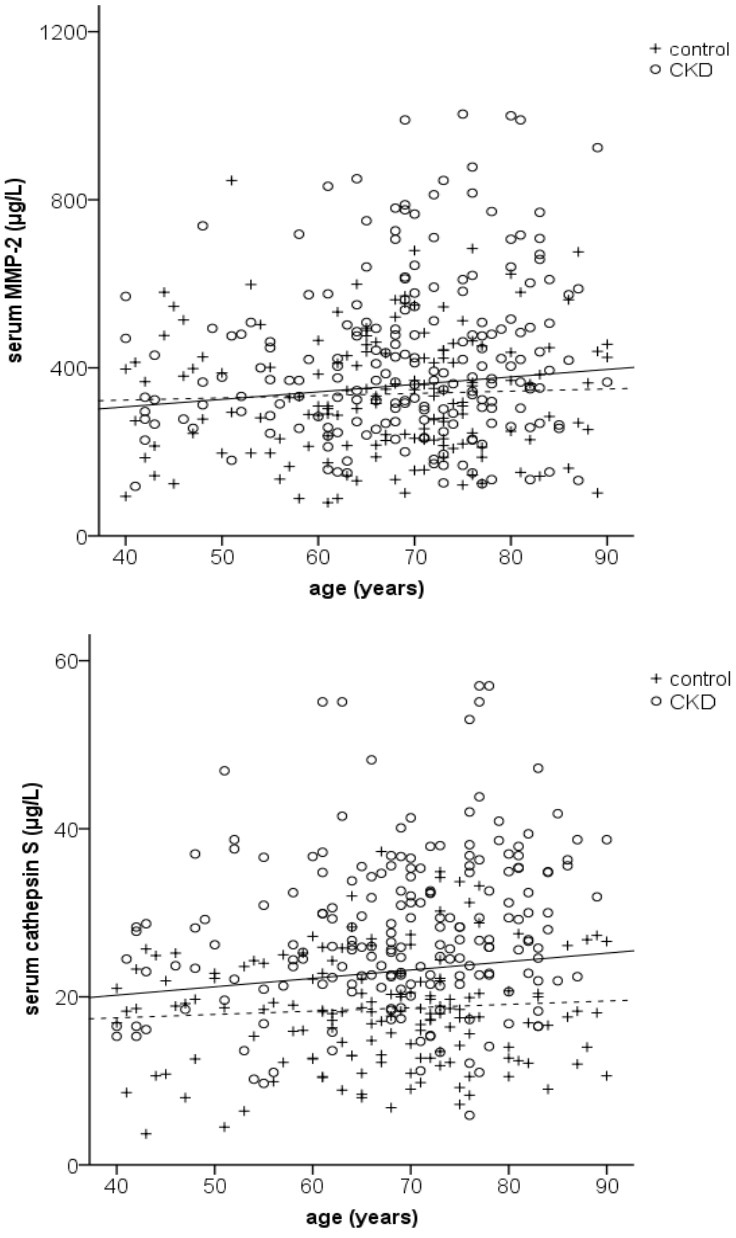

Figure S4. Linear regression of age-related variation in (A) serum MMP-2 concentration in stage 3 & 4 CKD patients (open circles, n=200, B=1.78 µg/L/year) and healthy controls (crosses, n=152, B=0.53 µg/L/year), (B) serum cathepsin S concentration in stage 3 & 4 CKD patients (open circles, n=200, B=0.17 µg/L/year) and healthy controls (crosses, n=152, B=0.05 µg/L/year). CKD = solid line; control = dashed line

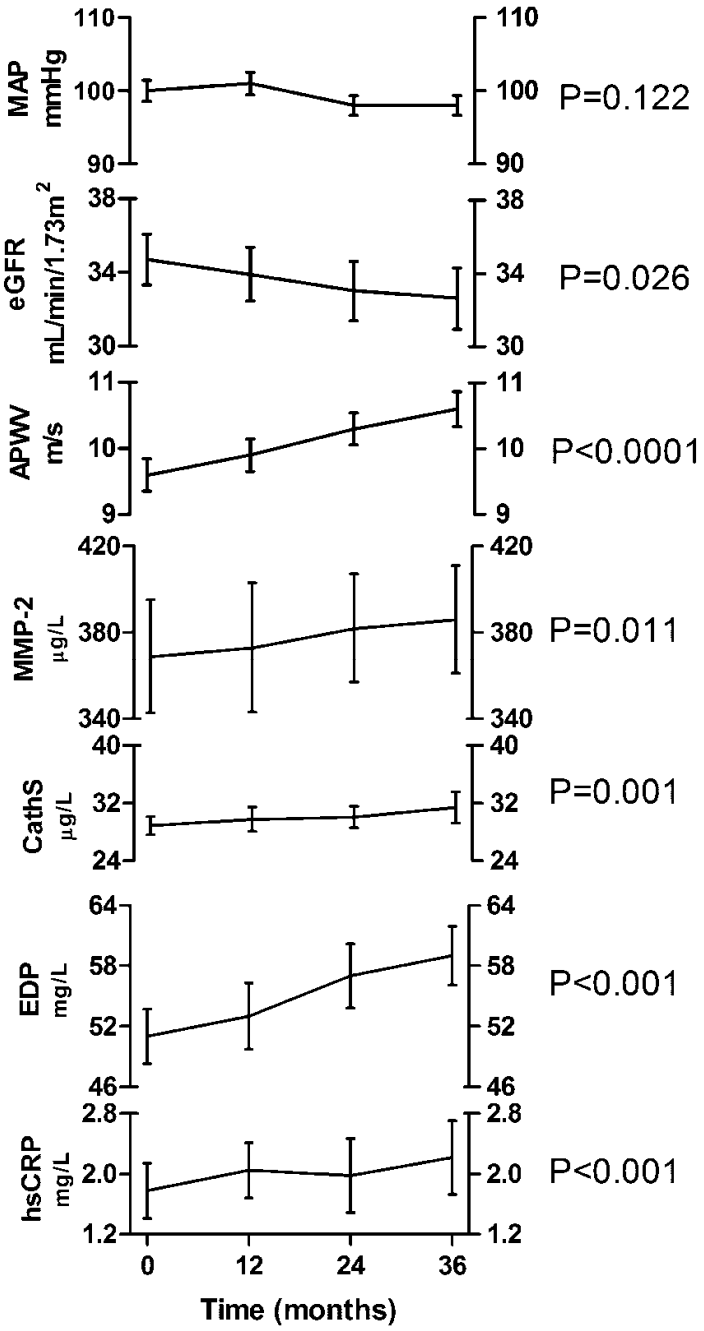

Figure S5. Serial changes in mean MAP, eGFR, APWV, serum MMP-2, cathepsin S and EDP concentration over 36 months in 65 patients with stage 3 & 4 CKD (error bars = SEM, P for trend). Scaling factor (x0.8) applied to APWV values.

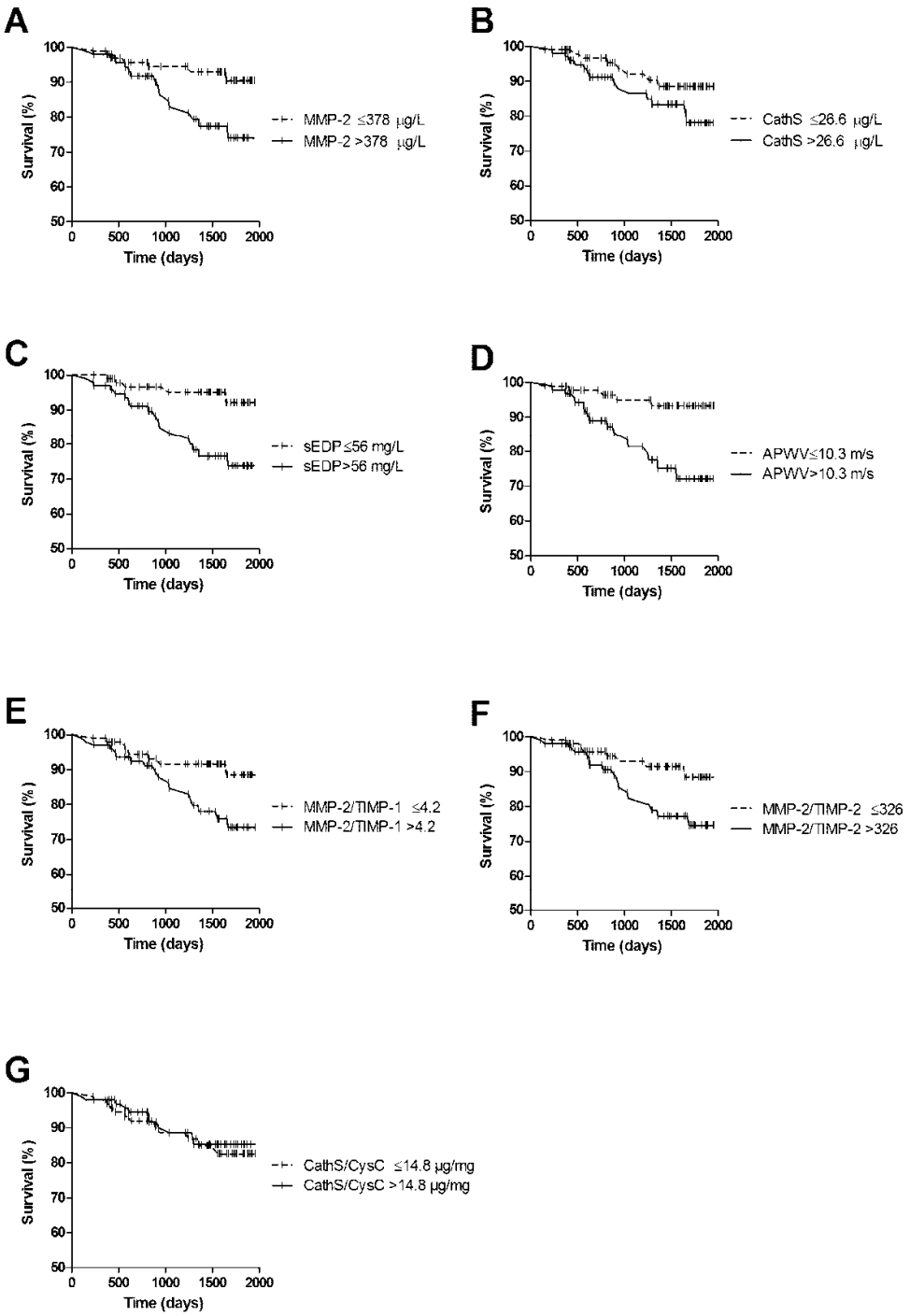

Figure S6. Unadjusted Kaplan-Meier survival curves showing all-cause mortality according to median (A) MMP-2, (B) cathepsin S, (C) EDP (D) APWV (E) MMP-2/TIMP-1, (F) MMP-2/TIMP-2 and (G) CathS/CysC in 200 patients with stage 3 & 4 CKD.