Effect of Circulating Soluble Receptor for Advanced Glycation End Products (sRAGE) and the Proinflammatory RAGE Ligand (EN-RAGE, S100A12) on Mortality in Hemodialysis Patients

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Background and objectives: The soluble receptor of advanced glycation end products (sRAGE) may exert anti-inflammatory protective roles on the vasculature. In contrast, the RAGE ligand S100A12 (also known as EN-RAGE) contributes to inflammation and the development of atherosclerosis in animal models. Whether alterations at this level contribute to the increased mortality observed in patients on dialysis is currently unknown.

Design, setting, participants, & measurements: Prospective study including 184 prevalent hemodialysis patients and 50 healthy controls matched for age and gender. Plasma concentrations of S100A12 and sRAGE were studied in relation to risk profile and mortality after a median follow-up period of 41 months.

Results: S100A12 and sRAGE levels were significantly elevated in hemodialysis patients compared with healthy controls. S100A12 had a strong positive correlation with C-reactive protein and IL-6, whereas sRAGE negatively associated with C-reactive protein. S100A12, but not sRAGE, was independently and positively associated with clinical cardiovascular disease (CVD). During follow-up, 85 (33 cardiovascular-related) deaths occurred. Whereas sRAGE did not predict mortality, S100A12 was associated with both all-cause (per $\log_{10}$ ng/ml hazard ratio [HR] 1.93, 95% confidence interval [CI] 1.18 to 3.15) and CVD-related (HR 3.23, 95% CI 1.48 to 7.01) mortality, even after adjustment for age, sex, vintage, and comorbidities. Further adjustment for inflammation made the predictive value of S100A12 disappear for all-cause mortality, but still persisted in CVD-related mortality.

Conclusions: Circulating S100A12 and sRAGE are both elevated in hemodialysis patients. However, only S100A12 associates with mortality, partly explained by its links with inflammation.

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Circulating plasma advanced glycation end products (AGEs) accumulate in chronic kidney disease (CKD), likely because of renal retention (1,2). The binding of ligands including AGEs to its receptor (RAGE), a member of the Ig superfamily, results in rapid and sustained cellular activation and gene transcription, leading to induction of inflammatory response that may contribute to atherosclerotic processes and a variety of microvascular and macrovascular complications (3–5). RAGE accumulates in disorders such as diabetes and CKD (6,7) and exists in several variants, that is, as a transmembrane receptor on the cell surface (the most abundant) or as isoform lacking the N-terminal domain (N-truncated variant) or the C-terminal (transmembrane) domain (C-truncated variant or soluble RAGE [sRAGE]) (8,9). sRAGE is shed from cell-surface RAGE and effectively binds circulating peptide and protein ligands including AGEs, thus antagonizing downstream RAGE signaling at the tissue level (10,11). Whereas sRAGE levels are elevated in CKD patients (12), they are also inversely associated with intima-media thickness (IMT) and plaque number (13). sRAGE covers both endogenous secretory RAGE (esRAGE) and cleaved-type soluble RAGE (14), and esRAGE was reported as an inverse predictor of cardiovascular death in hemodialysis (HD) patients (15).

S100A12, also known as extracellular newly identified RAGE binding protein (EN-RAGE), is a ligand for RAGE, which regulates monocyte migration and induces proinflammatory cytokine production in macrophages (16–18). S100A12-RAGE interaction drives proinflammatory gene transcription through NF-$\kappa\beta$ activation (19). In human coronary atherosclerotic plaques, a strong expression of S100A12 in smooth muscle cells and macrophages was reported (20), involving altogether S100A12 binding in the development of atherosclerosis. Similar to sRAGE levels, S100A12 levels are

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also elevated in patients with CKD and positively associated with IMT (21).

Because cardiovascular complications are the most common cause of death in HD patients (22,23), it is important to understand the complex processes that may occur and lead to cardiovascular disease (CVD). In this study, we evaluated the effect of plasma concentrations of S100A12 and sRAGE on mortality and also possible links with clinical phenotype, including inflammatory, nutritional status, and existing comorbidities in a well-characterized cohort of prevalent HD patients.

Materials and Methods

Patients and Study Design

This study was performed at five dialysis units in Stockholm, and one at the Uppsala Academic Hospital in Uppsala, Sweden. This is a *post hoc* analysis of baseline data arising from a study originally aiming at investigating the variability of inflammatory markers in HD patients. The protocol has been previously described in more detail and patient recruitment took place between October 2003 and March 2004 (24). Of the 224 prevalent patients included in the study and followed for assessment of overall and CVD mortality, 40 patients were excluded because either S100A12 or sRAGE was not measured because of lack of stored plasma. In addition, we included 50 healthy randomly selected individuals with normal renal function for comparison regarding S100A12 and sRAGE levels. These control patients were investigated according to a protocol similar to the hemodialysis patients group. Selection of patients in the Stockholm region was performed by Statistics Sweden. Diagnosed diabetes or CVD and unwillingness to participate were the only exclusion criteria. The study protocols were approved by the Ethics Committee of the Karolinska Institutet Hospital and Uppsala University Hospital. Signed informed consent was obtained from all patients and controls before inclusion. Immediately after recruitment, each patient's medical chart at baseline was thoroughly reviewed by a single clinician, who extracted data on the history of CVD, diabetes, and other comorbid conditions. Survival was determined from the day of examination and during a median follow-up period of 41 (interquartile range 23 to 48) months. There was no loss of follow-up of any patient. Causes of death were collected from medical records. Cardiovascular mortality was defined as death resulting from coronary heart disease, stroke, or complicated peripheral vascular disease.

Nutritional Status and Laboratory Analysis

Height was obtained from the patient's chart. Body weight and body mass index (BMI) were taken on a dialysis day. Subjective global assessment was used to obtain a clinical estimate of malnutrition, as described previously (25). For the purposes of this study, malnutrition (or rather protein-energy wasting [PEW]) was defined as a subjective global assessment score >1. Blood samples were collected before a HD session after the longest interdialytic period. Plasma and serum were separated and kept frozen at –70°C if not analyzed immediately. Plasma concentrations of S100A12 (CircuLex S100A12/EN-RAGE ELISA kit; CycLex Co., Ltd., Nagano, Japan) and sRAGE (Human RAGE Immunoassay; R&D Systems, Inc., Minneapolis) were measured using a commercially available ELISA kit according to the instructions of the manufacturer. Serum concentrations of IL-6 (Siemens Medical Solutions Diagnostics, Los Angeles) were quantified by immunometric assays on an Immulite Analyzer according to the instructions of the manufacturers. Circulating levels of albumin, creatinine, total cholesterol, LDL cholesterol, calcium, phosphate, white blood cells, and high-sensitivity C-reactive protein (CRP) were analyzed using certified methods at the Department of Laboratory Medicine at Karolinska University Hospital or Uppsala Academic Hospital.

Statistical Analyses

All variables were expressed as the mean ± SD or median and interquartile range (25th to 75th percentiles), unless otherwise indicated. Statistical significance was set at the level of $P < 0.05$. Comparisons between two groups were assessed with the Mann-Whitney U test or χ^2 test, as appropriate. Spearman rank correlation analysis was used to determine associations between S100A12 or sRAGE with selected parameters. Multivariate regression analyses were used to assess independent predictors of S100A12 and sRAGE, whereas logistic regression approaches were used to assess determinants of existing CVD. Survival analyses were made with the Cox proportional hazard model. The univariate and multivariate Cox regression analyses are presented as hazard ratios (HR) and 95% confidence intervals (CI). In the case of S100A12, and to prevent overfitting in small sample sizes (monotone likelihood), a shrinkage factor with Firth correction was applied (26,27). All statistical analyses were performed using statistical software SAS version 9.2 (SAS, Cary, NC).

Results

Clinical Characteristics and Univariate Correlates of S100A12 and sRAGE Levels

Clinical characteristics of the patients included in the study are summarized in Table 1. Forty-five (24%) of the patients had diabetes mellitus, whereas 118 (64%) had a clinical history of CVD. Patients were treated with HD three times a week (4 to 5 hours per session). Plasma concentrations of S100A12 and sRAGE were significantly higher in HD patients as compared with healthy controls (Figure 1, Table 1), but did not differ between those who had diabetes and those who did not. In univariate analysis (Table 1), there was a positive correlation between S100A12 and CRP (Figure 2A) and a negative correlation between sRAGE and CRP (Figure 2B). Also, S100A12 levels were positively associated with a clinical history of CVD, PEW, and markers of inflammation (white blood cell count and IL-6), but negatively associated with dialysis vintage. Furthermore, female patients had higher plasma concentrations of sRAGE than male patients (4.4 [2.9 to 7.1] *versus* 3.5 [2.4 to 5.4] ng/ml, $P = 0.020$) and sRAGE levels negatively associated with BMI, CRP, and IGF-1, but did not relate to the S100A12 levels (Table 1).

Multivariate Correlations

We performed a multivariate regression analysis of contributing factors to explain S100A12 levels, testing whether the associations between S100A12 and CVD, PEW, or inflammation were a reflection of aging or were confounded by sex. Clinical history of CVD and inflammation, but not PEW, were independently associated with S100A12 levels (Table 2A). Table 2B shows the results of multivariate regression analysis of factors predicting sRAGE; sex, BMI, and inflammation were independent contributors to sRAGE values.

Table 1. General characteristics of hemodialysis patients and healthy controls and univariate associations with S100A12 and sRAGE in patients

Variables	Healthy Controls ($n = 50$)	Hemodialysis Patients ($n = 184$)	ρ[b]	
			S100A12	sRAGE
S100A12, ng/ml	6.7 (4.6 to 10.0)[d]	14.7 (8.8 to 32.5)[a]		
sRAGE, ng/ml	1.3 (0.8 to 1.7)	3.9 (2.7 to 6.0)[a]	−0.056	
Age, years	63 (58 to 70)	67 (51 to 74)	0.007	0.042
Men, %	31 (62%)[f]	101 (55%)	0.036	−0.171*
Dialysis vintage, months		29 (14 to 55)	−0.150*	0.111
Diabetes mellitus, %	0 (0%)	45 (24%)[a]	0.033	−0.017
Clinical CVD, %	0 (0%)	118 (64%)[a]	0.178*	0.013
BMI, kg/m^2	25.4 (±3.9)[e]	24.8 (±5.3)	0.041	−0.259***
PEW,[c] %	2 (4%)	84 (46%)[a]	0.149*	0.038
s-Albumin, g/L	39.0 (±2.8)	35.0 (±4.2)[a]	−0.119	0.014
s-Creatinine, μmol/L	79 (±15)	786 (±207)[a]	−0.056	−0.126
Total cholesterol, mmol/L	5.2 (±0.8)	4.3 (±0.9)[a]	0.060	0.081
LDL cholesterol, mmol/L	3.3 (±0.8)	2.6 (±0.8)[a]	0.081	0.038
Calcium, mmol/L	2.3 (±0.1)	2.5 (±0.2)[a]	0.062	0.053
Phosphate, mmol/L	1.0 (±0.2)	1.9 (±0.6)[a]	0.078	−0.041
White blood cell count, 10^9/L	6.1 (±1.9)	8.0 (±2.5)[a]	0.376***	0.026
CRP, mg/L	1.2 (0.6 to 2.6)	6.4 (2.5 to 19.5)[a]	0.426***	−0.198**
IL-6, pg/ml	1.4 (1.0 to 2.7)	8.5 (4.8 to 15.0)[a]	0.391***	−0.065

[a]Significantly different ($P < 0.05$) from healthy individuals as assessed by Mann-Whitney U or χ^2 test.
[b]Univariate correlations as assessed by Spearman's rank correlation analysis. Asterisks denote statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[c]Defined as subjective global assessment >1.
[d]Median value; 25th to 75th percentile shown in parentheses (all such values).
[e]Mean ± SD (all such values).
[f]Prevalence, shown in number and percentage (all such values).

Higher S100A12 Levels in Patients with CVD

Patients with a clinical history of CVD had higher S100A12 levels as compared with those with no CVD history (17.5 [9.2 to 34.7] *versus* 11.6 [7.7 to 27.9] ng/ml, $P = 0.016$). In a logistic regression model, this association remained and was independent of age, sex, and diabetes (Table 3). However, for circulating sRAGE, there was no difference according to CVD stratification (4.2 [2.7 to 5.9] *versus* 3.5 [2.6 to 6.5] ng/ml, $P = 0.860$).

Higher S100A12 Levels in Patients with Poor Outcome

During the follow-up period, 85 (46%) patients died, 33 (39%) from CVD-related causes. The baseline S100A12 levels in the patients who died from CVD were higher than those in patients who died from non-CVD–related causes (26.5 [14.0 to 42.6] *versus* 14.9 [8.8 to 29.0] ng/ml, $P < 0.001$, $n = 33$ of 52). Cox proportional hazard crude analyses showed that patients with higher levels of S100A12 had an increased mortality by all causes (HR per $\log_{10}$ ng/ml 1.93, 95% CI 1.18 to 3.15, $P = 0.009$) and by CVD-related causes (HR 3.23, CI 1.48 to 7.01, $P = 0.003$) (Table 4A). This difference persisted after adjustment for age, sex, dialysis vintage, and baseline comorbidities. Further adjustment for inflammation made the predictive value of S100A12 on all-cause mortality disappear, but still persisted in CVD-related mortality. Crude Cox proportional hazard crude analyses showed that plasma sRAGE did not associate with outcome (Table 4B). After multivariate adjustment, hazard ratios were still statistically insignificant but increased in magnitude.

Discussion

This study shows, we believe for the first time in CKD and in any other disease, that S100A12 concentration was a direct predictor of all-cause and CVD mortality, even after adjustment for confounders. S100A12, as cofacilitator/initiator of the AGE-RAGE inflammatory response (19), also showed a strong positive correlation with systemic inflammation. Consequently, adjustment for inflammation significantly attenuated the association of S100A12 with all-cause mortality, but not with cardiovascular mortality. This study also shows that although sRAGE negatively associated with inflammation, it did not relate to CVD or outcome. Altogether, this study identifies the predictive value of S100A12 on (cardiovascular) mortality and provides further clinical evidence on its possible role in the development of CVD.

In agreement with previous reports, our study confirms that both plasma concentrations of S100A12 (21) and sRAGE (13,28) are elevated in patients with CKD as compared with those in healthy individuals. However, our observation opposes the reports in individuals with type 2 diabetes, where S100A12 was

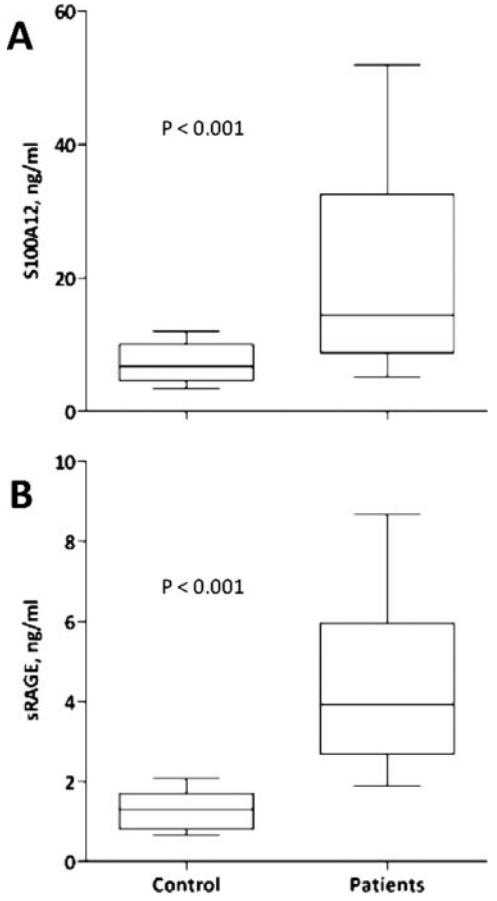

Figure 1. Plasma concentration of S100A12 (A) and sRAGE (B) in healthy controls and in prevalent hemodialysis patients. Differences were assessed by Mann-Whitney *U* test.

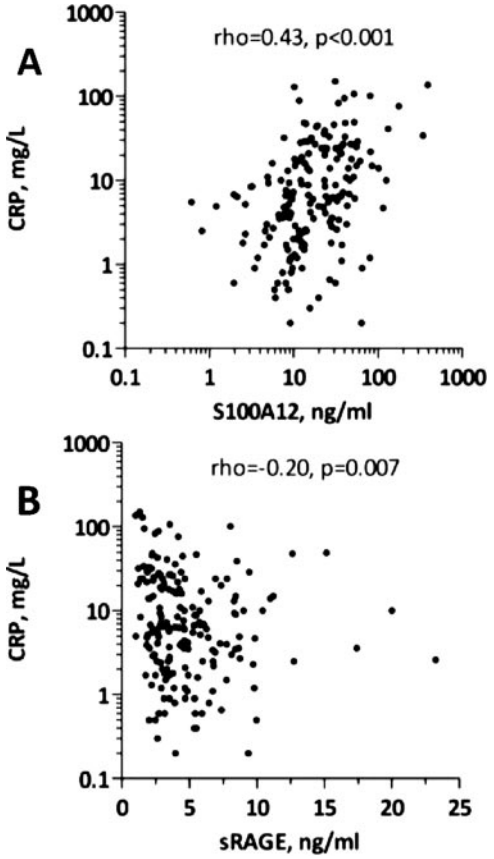

Figure 2. Univariate correlations between CRP and S100A12 (A) and sRAGE (B).

increased (29,30) and sRAGE decreased (30) as compared with those in healthy patients. Also, sRAGE was significantly lower in patients with angiographically proven coronary artery disease than in age-matched healthy controls (31). sRAGE levels increase with declining renal function and substantially decrease after renal transplantation (28,32). Although we are still ignorant of the precise mechanism whereby sRAGE increases in CKD, it may well be a counter-regulatory mechanism activated to counteract the vasculotoxic effect of AGEs accumulation in uremia.

We also show that whereas S100A12 was positively correlated with CRP in HD patients (21), sRAGE had a negative correlation (13,28). These data are compatible with the hypothesis that high sRAGE may act as a vasculoprotective factor in this population, whereas S100A12 may be deleterious as a decoy of the AGE-RAGE activation of the inflammatory response. Additionally, S100A12 related to IL-6 in our study, in agreement with an animal report in transgenic mice overexpressing human S100A12 that demonstrated the mediation of S100A12 in the process of IL-6 production and aortic wall remodeling (33). Several clinical studies have consistently shown the association between circulating S100A12 and atherosclerosis or CVD risk (30,34). The same is true for HD patients, where Mori *et al.* (19) reported a direct association between plasma S100A12 and IMT. However, there have been conflicting reports regarding sRAGE: Although some studies associated sRAGE and atherosclerosis in non-CKD populations (30,31) and recently by Basta *et al.* (13) in patients with CKD not yet on dialysis, others could not confirm this finding (14,28). Our study adds to this evidence that whereas S100A12 significantly associated with clinically evident CVD in univariate and multivariate analysis, sRAGE did not.

AGEs are generated as a result of chronic hyperglycemia and enhanced oxidative stress. The binding of these proteins with their receptors, such as RAGE, induce oxidative stress, inflammation, and extracellular matrix accumulation, all effects that eventually translate into accelerated plaque formation and atherogenesis (3,4). Experimentally, AGEs binding to sRAGE prevents proinflammatory effects by acting as a decoy receptor (9). However, sRAGE levels were not related to mortality in our study, confirming a previous report in a small HD cohort (28). Altogether, it seems therefore unlikely that the measurement of sRAGE is useful in clinical practice to identify patients at a higher atherosclerotic or mortality risk. However, the issue remains to be tested in a specifically designed clinical trial.

The chief finding of this study is that S100A12 levels predict all-cause and CVD-related mortality. Although there is still insufficient knowledge about the mechanisms by which S100A12 may induce disease and worse outcome, recent studies suggest that S100A12 regulates monocyte migration and

Table 2. Multivariate regression model predicting for S100A12 (top) and sRAGE (bottom) in 184 hemodialysis patients

Parameter	Parameter Estimate	Standard Error	*P*
S100A12[a]			
intercept	0.981	0.073	<0.0001
age, ≥67 years	−0.052	0.062	0.4
gender, men	−0.007	0.061	0.9
diabetes mellitus, presence	−0.007	0.069	0.9
clinical history of CVD, presence	0.146	0.063	0.02
PEW, presence	0.041	0.062	0.5
inflammation, presence	0.383	0.061	<0.0001
sRAGE[b]			
intercept	0.747	0.041	<0.0001
age, ≥67 years	−0.038	0.037	0.3
gender, men	−0.107	0.037	0.004
diabetes mellitus, presence	−0.006	0.045	0.9
BMI, ≥26.3 kg/m^2	−0.102	0.042	0.01
inflammation, presence	−0.090	0.039	0.02

[a]The adjusted r^2 of the model was 0.20. Age was dichotomized according to the median value. Inflammation was defined as CRP >10 mg/L.
[b]The adjusted r^2 of the model was 0.10. Age was dichotomized according to the median value. Inflammation was defined as CRP >10 mg/L, and an increased BMI was defined according to the 66th percentile of our sample distribution.

Table 3. Odds ratios and 95% CI for factors predicting the presence of CVD at time of inclusion in 184 hemodialysis patients

Parameter	Odds Ratio (95% CI)	*P*
Intercept		0.011
Age, ≥67 years	3.15 (1.63 to 6.10)	0.0007
Gender, men	1.41 (0.74 to 2.69)	0.3
Diabetes mellitus, presence	1.55 (0.73 to 3.31)	0.3
S100A12, per log ng/ml	2.74 (1.26 to 5.96)	0.01

The adjusted r^2 of the model was 0.11. Age was dichotomized according to the median value.

induces proinflammatory cytokine production in macrophages (16–18). In addition, engagement of RAGE to S100A12 drives proinflammatory gene transcription through activation of NF-$\kappa\beta$ (19) and upregulates both vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 synthesis (16,17). Therefore, our finding that adjustment for systemic inflammation made the predictive value of S100A12 on all-cause mortality statistically insignificant is consistent with its reported mechanisms of action. However, the predictive value on CVD outcome still remained, leading us to speculate that S100A12 may lead to atherogenesis and mortality by yet uncharacterized non-proinflammatory mechanisms. In accordance, a recent report by Hofman *et al.* (33) demonstrates that S100A12 reduces vascular smooth muscle cell proliferation and increases cytosolic H_2O_2 production via NADPH oxidase system. Theoretically, possible therapeutic strategies targeting S100A12 may raise considerable interest. Obviously, further basic studies are warranted to clarify the role of this interesting ligand. We could not find, however, a statistically significant effect of sRAGE on outcome, although the adjusted HR for CVD-related mortality in our study was high in magnitude. Thus, we cannot exclude the possibility that a larger sample size would have allowed us to observe an effect at this level. Nonetheless, Kalousová *et al.* (28) did not find an association between sRAGE and mortality either in 261 HD patients followed for 30 months. The direction of this association would agree with the earlier report by Koyama *et al.* (15), who found that esRAGE was an inverse predictor of cardiovascular death.

Some limitations of this study should be acknowledged, starting with its cross-sectional design, which limits the ability to establish causal relationships, and the fact that fatal cardiovascular events are extracted from patient records and not confirmed by autopsies, probably underestimating the true prevalence of cardiac end points. Furthermore, as we studied a cohort with a relatively small number of patients, we cannot draw solid conclusions and our data need to be confirmed in larger materials. Notwithstanding these possible limitations, the extensive phenotyping, including inflammatory biomarkers, comorbidities, and deaths, is a strength of our study, which allowed us to ensure a more unbiased estimate of observed relations.

In conclusion, S100A12 and sRAGE are elevated and have opposite associations with inflammation in prevalent HD patients. S100A12 is associated with a clinical history of CVD and is a strong predictor of mortality. The association of S100A12 with mortality may be explained, at least in part, by its proinflammatory effects. Longitudinal observations and intervention studies are warranted to establish whether this link is causal in nature.

Table 4. Hazard ratios for S100A12 (A) and sRAGE (B) levels with all-cause and CVD-related mortality in 184 hemodialysis patients

Model	Covariates	All-Cause Mortality		CVD Mortality	
		HR (95% CI)	*P*	HR (95% CI)	*P*
(A) S100A12					
1	Crude (per $\log_{10}$ ng/ml)	1.93 (1.18 to 3.15)	0.008	3.43 (1.54 to 7.41)	0.002
2	1 + age and sex	2.02 (1.22 to 3.27)	0.005	3.34 (1.53 to 7.03)	0.002
3	2 + dialysis vintage, diabetes, and baseline CVD	1.87 (1.12 to 3.13)	0.01	3.03 (1.32 to 6.98)	0.009
4	3 + IL-6 (per pg/ml)	1.67 (0.97 to 2.87)	0.06	3.33 (1.39 to 7.86)	0.006
(B) sRAGE					
1	Crude (per $\log_{10}$ ng/ml)	1.03 (0.44 to 2.37)	0.94	1.34 (0.34 to 5.05)	0.67
2	1 + age and sex	1.40 (0.58 to 3.34)	0.45	2.11 (0.51 to 8.67)	0.31
3	2 + dialysis vintage, diabetes, and baseline CVD	1.46 (0.62 to 3.41)	0.38	2.02 (0.50 to 7.95)	0.32
4	3 + IL-6 (per pg/ml)	1.67 (0.71 to 3.92)	0.24	2.24 (0.55 to 9.04)	0.26

Indicated are crude HRs or with various degrees of adjustment (models 2 to 4) for all-cause and CVD-related mortality according to S100A12 and sRAGE levels. Categories for age and dialysis vintage were calculated according to the median value of the group. To correct for possible overfitting of the model, a shrinkage factor with Firth correction was applied in (A).

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