

Total Soluble and Endogenous Secretory Receptor for Advanced Glycation End Products as Predictive Biomarkers of Coronary Heart Disease Risk in Patients With Type 2 Diabetes

An Analysis From the CARDS Trial

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OBJECTIVE—Circulating levels of soluble receptor for advanced glycation end products (sRAGE) likely comprise both a secreted isoform (esRAGE) and wild-type RAGE cleaved from the cell membrane. Both sRAGE and esRAGE have been proposed as biomarkers of cardiovascular disease (CVD), but prospective data are limited. We examined the relationship of sRAGE and esRAGE to incident coronary heart disease (CHD) and stroke in type 2 diabetic patients followed for 3.9 years in a trial of atorvastatin: the Collaborative Atorvastatin Diabetes Study (CARDS).

RESEARCH DESIGN AND METHODS—We used a nested case-control design sampling all incident cases of CVD with available plasma and randomly selecting three control subjects, who were free of CVD throughout follow-up, per case. Analysis was by Cox regression with adjustment for treatment allocation and relevant covariates.

RESULTS—sRAGE and esRAGE were strongly correlated ($\rho = 0.88$) and were both higher in those with lower BMI ($P < 0.001$), higher adiponectin ($P < 0.001$), lower estimated glomerular filtration rate ($P = 0.009$), and white ethnicity ($P < 0.001$). Both sRAGE and esRAGE were associated with incident CHD events, independently of treatment allocation and the above factors; hazard ratio (HR) = 1.74 (95% CI 1.25–2.41; $P = 0.002$) for a doubling of the sRAGE level; HR = 1.45 (1.11–1.89; $P = 0.006$) for a doubling of the esRAGE level. There was no significant association with stroke; HR for sRAGE = 0.66 (0.38–1.14). Atorvastatin, 10 mg daily, did not alter sRAGE.

CONCLUSIONS—Higher levels of sRAGE and esRAGE are associated with incident CHD but not stroke in type 2 diabetes. *Diabetes* 60:2379–2385, 2011

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Receptor for advanced glycation end products (RAGE) is a cell-surface molecule that binds many ligands, including advanced glycation end products (AGEs) (1,2). This binding results in diverse responses, including altered gene expression and cell migration and proliferation, in pathways that are considered to play a pivotal role in the pathogenesis of atherosclerosis, heart failure, and other diabetes complications. The involved pathways relevant to atherosclerosis are diverse; for example, blocking ligand binding to RAGE in mice reduced diabetes-induced inflammation and atherosclerotic plaque formation and reduced vascular smooth muscle proliferation and migration and extracellular matrix production in response to injury (3). A circulating soluble form of RAGE has been proposed as a potentially useful biomarker of cardiovascular disease (CVD) in diabetes. This soluble RAGE (total sRAGE) likely comprises both the extracellular domain of wild-type, full-length RAGE, which results from proteolytic cleavage at the cell surface, and an endogenous secreted isoform lacking a transmembrane domain (RAGE-VI or esRAGE) that can also be measured separately (4–6). RAGE antagonists are in clinical development as therapeutics for diabetes complications and Alzheimer disease. An important question, therefore, is the usefulness of circulating total sRAGE (hereafter termed sRAGE) and esRAGE as potential biomarkers of diabetes complications and need for therapy.

The published literature on esRAGE and sRAGE prediction of complications in patients with diabetes is sparse and conflicting (6–8). sRAGE levels have been found to be higher in coronary disease cases, or those with higher atherosclerotic burden, than control subjects in some studies (7–12) but lower in others (13). esRAGE levels have been mostly reported to be lower in case subjects, or those with greater atherosclerosis burden, than control subjects (11,14–16). However prospective data are essential to fully understand the relationship between sRAGE and CVD. Two recent prospective studies showed that higher levels of sRAGE were associated with CVD in type 1 diabetes (8,12). There are no prospective studies that have examined both esRAGE and sRAGE as predictors of CVD in type 2 diabetes with adjustment for other risk factors. There is also need for more clinical data on the relationship of

circulating esRAGE and sRAGE with other factors in diabetes.

The primary purpose of this analysis was to examine the relationship of serum esRAGE and sRAGE to incident cardiovascular events. We also explored the relationship of esRAGE and sRAGE with each other and with other risk factors in type 2 diabetes. We examined these questions using samples and data from a randomized controlled clinical trial of statin therapy in type 2 diabetic patients without prior CVD (the Collaborative Atorvastatin Diabetes Study [CARDS]) (17).

RESEARCH DESIGN AND METHODS

The design of the CARDS trial has been reported previously (17). The trial was conducted in 132 clinical centers in the U.K. and Ireland. In it, 2,838 patients with type 2 diabetes without previous CVD were randomized to receive either atorvastatin (10 mg daily) or placebo. Patients were ineligible if they had any past history of myocardial infarction, angina, coronary vascular surgery, cerebrovascular accident, or severe peripheral vascular disease. We checked eligibility against the patients' clinical notes. The median follow-up duration in the trial was 3.9 years. The primary end point was major CVD events comprising myocardial infarction including silent infarction, unstable angina, acute coronary heart disease (CHD) death, resuscitated cardiac arrest, coronary revascularization procedures, or stroke. An independent end point committee reviewed all reported cardiovascular events and deaths and classified them according to criteria specified in the end point protocol. We used a nested case-control design sampling all those incident cases of CVD ($n = 210$) during follow-up in CARDS in whom a valid baseline prerandomization serum sample was available and randomly selecting three control subjects per case subject from those who completed the trial without a CVD event, stratified by treatment allocation. A valid sample for sRAGE and esRAGE was finally available in 167 (80%) of all cases, and the control subjects with sRAGE data (551) comprised 20% of all those without CVD during follow-up.

Using prerandomization samples, serum sRAGE was measured using the R&D Systems Quantikine Immunoassay (Minneapolis, MN) that is specific for the extracellular domain of human RAGE. The assay coefficient of variation was 4.4%. esRAGE was measured using the B-Bridge ELISA (B-Bridge International, Cupertino, CA). This assay specifically measures the esRAGE/RAGEv1 protein only due to the use of an antibody directed against the unique COOH-terminal sequence of RAGE-v1 and does not cross-react with other

potential forms of sRAGE. The coefficient of variation was 6%. The correct terminology for this splice variant is RAGE-v1(6) but we retain the term esRAGE here as that is how the assay is described by the manufacturers. Sufficient sample was available for sRAGE and esRAGE measurement for 715 and 780 participants, respectively. As these two assay systems for esRAGE and sRAGE are constituted by individual antibodies and different standard proteins, they are not directly comparable. Nonetheless, some information can be gained by examining determinants of their relative amounts. To evaluate whether atorvastatin affects sRAGE we also measured sRAGE in a posttreatment sample taken at 1 year of follow-up. We did not measure esRAGE at follow-up.

Statistical analysis used multiple linear regression with covariate adjustment to examine cross-sectional determinants of sRAGE and esRAGE at baseline. Analysis of prediction of CVD events was by Cox regression with adjustment for treatment allocation and relevant covariates. The validity of the proportional hazards assumption was confirmed by a test for interaction of the hazard ratio (HR) with time. Given the skewed distribution of sRAGE and esRAGE (see Fig. 2), these were log transformed in the analysis. Analyses were conducted using STATA 10 MP for Linux.

RESULTS

Table 1 shows the baseline characteristics of those study participants with available baseline sRAGE data who went on to become CVD cases compared with the control group selected who did not develop CVD. Those who developed CVD were older, were more likely to be a current or ex-smoker, had albuminuria, were male, and had slightly higher LDL-C. Further analyses for CVD risk adjusted for these factors. Among the 167 CVD cases, there were 128 CHD and 44 stroke events (5 people having both). Similar differences in baseline characteristics were seen for CHD cases versus control subjects as for CVD cases versus control subjects.

Figure 1 summarizes the main relationships we found between other factors and sRAGE. A very similar pattern of relationships was found for esRAGE. Neither sRAGE nor esRAGE differed significantly with age or by sex. However, although the numbers of nonwhite participants was low ($n = 20$ African Caribbean; $n = 25$ South Asians), there were marked ethnic differences, with much lower

TABLE 1
Baseline characteristics of incident CVD cases and control subjects

	CVD cases ($n = 167$)	Control subjects ($n = 551$)	<i>P</i> value for difference*
Mean (SE)			
Age (years)	64 (0.53)	61 (0.34)	<0.001
BMI (kg/m^2)	28.9 (0.25)	28.8 (0.15)	0.06
Systolic BP (mmHg)	146 (1.20)	144 (0.68)	0.63
LDL-C (mmol/L)	3.2 (0.06)	3.0 (0.03)	0.004
HDL-C (mmol/L)	1.37 (0.02)	1.4 (0.01)	0.52
HbA _{1c} (%)	7.9 (0.10)	7.8 (0.06)	0.23
Median (interquartile range)			
sRAGE (pg/mL)	1,404 (1,097–1,877)	1,404 (1,057–1,777)	0.096
Median esRAGE (pg/mL)	330 (240–460)	330 (240–430)	0.39
Diabetes duration (years)	8 (4–12)	6 (3–11)	0.25
Percentages			
eGFR <60 mL/min/1.73 m ²	31	32	0.75
Men (%)	86	67	<0.001
Current smoker (%)	28	23	0.02
White (%)	93	93	0.49
Albuminuria (%)	17	10	0.039
Retinopathy (%)	41	27	0.003
Metformin (%)	46	46	0.29
Insulin (%)	17	20	0.53
RAS drugs (%)	40	48	0.08

RAS, renin angiotensinogen system. **P* values are for association of variables with CVD using logistic regression. Other than for age and sex, the logistic model adjusted for age, sex, and treatment allocation.

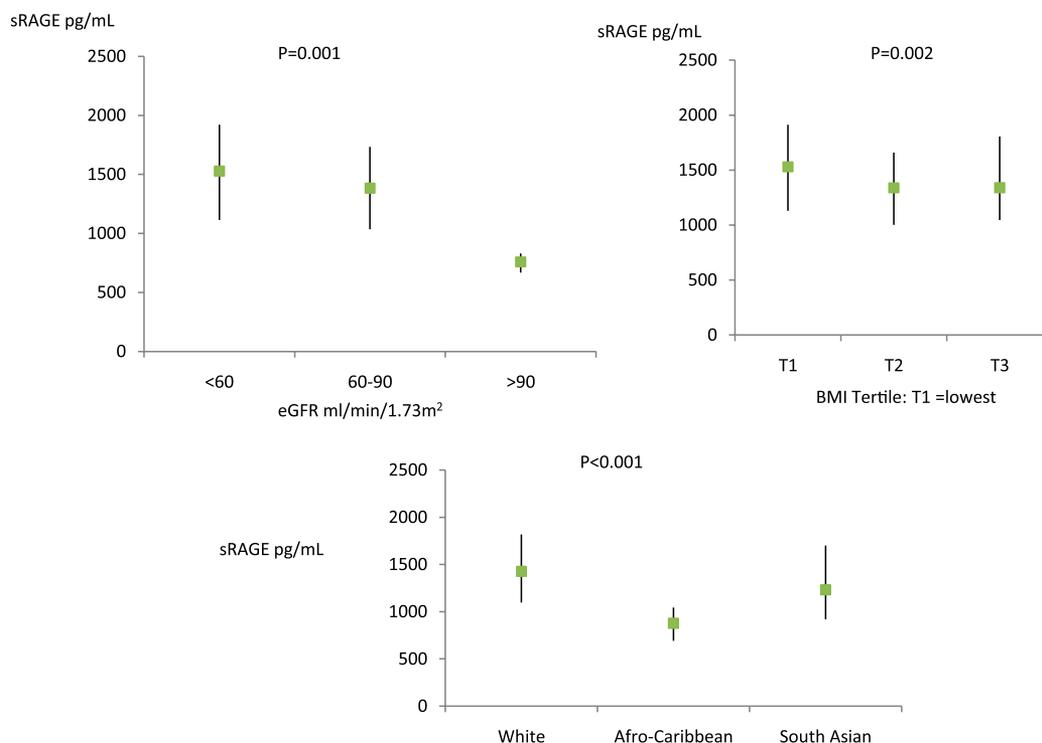


FIG. 1. Association of sRAGE with eGFR, BMI, and ethnicity. (A high-quality color representation of this figure is available in the online issue.)

sRAGE and esRAGE in those of African Caribbean origin compared with whites and South Asians having intermediate levels. Those with lower estimated glomerular filtration rate (eGFR) had higher sRAGE and esRAGE but there was no association with albuminuria (the prevalence of albuminuria in this study population was low, however [see Table 1]). There was a slight inverse association between HbA_{1c} and esRAGE (Spearman $\rho = -0.10$; $P = 0.006$) but not sRAGE (Spearman $\rho = -0.06$; $P = 0.09$). Those with lower BMI levels had higher levels of both sRAGE and esRAGE, and consistent with this, there was a positive correlation with adiponectin (Spearman $\rho = 0.14$; $P = 0.006$). In a linear regression model with the above variables considered simultaneously, sRAGE remained associated with BMI ($P = 0.007$) or adiponectin ($P < 0.001$), ethnicity ($P = 0.002$), and eGFR level ($P = 0.004$). esRAGE remained associated with BMI ($P < 0.001$) or adiponectin ($P < 0.001$), ethnicity ($P < 0.001$), HbA_{1c} ($P = 0.019$), and eGFR ($P = 0.05$). However, together these factors explained no more than 6 and 10% of the variance in sRAGE and esRAGE, respectively. There was no significant association between esRAGE and smoking, lipids, blood pressure, antihypertensive agents, diabetes duration, insulin, or oral diabetes drugs (data not shown). Neither was there any association with high-sensitivity C-reactive protein (Spearman $\rho = -0.02$; $P = 0.6$).

We explored the relationship of sRAGE to esRAGE. sRAGE and esRAGE were strongly correlated (Spearman $\rho = 0.88$), with a median ratio of sRAGE to esRAGE levels of 4.2 (ranging from threefold at the bottom 5% to sixfold at the top 5%). When we explored whether associations of patient characteristics to the ratio of sRAGE:esRAGE existed, we found that a higher ratio was associated with younger age ($P = 0.001$), higher HbA_{1c} ($P = 0.01$), being nonwhite ($P = 0.003$), and having a higher BMI ($P = 0.002$). (P values shown are for a model including these characteristics and sex simultaneously.)

We examined the relationship of sRAGE and esRAGE to cardiovascular events. As shown in Table 1, there was no apparent difference in esRAGE or sRAGE between cases and control subjects for CVD. This lack of association was confirmed in a Cox regression model adjusting for age, sex, and treatment (HR for sRAGE = 1.26; 95% CI 0.97–1.64; $P = 0.08$). However for CHD considered separately, both sRAGE and esRAGE were higher in cases than control subjects adjusted for age and sex. The median (interquartile range) sRAGE was 1,499 pg/mL (1,138–1,910) in cases and 1,395 pg/mL (1,046–1,777) in control subjects, and the median esRAGE was 340 pg/mL (250–460) in cases and 320 pg/mL (240–430) in control subjects. As shown in Table 2 and Fig. 2 this association of both sRAGE and esRAGE with CHD incidence was statistically significant, adjusted for age, sex, and treatment allocation ($P = 0.003$ for sRAGE and $P = 0.023$ for esRAGE; Table 2). In a Cox regression model adjusting further for potential CHD risk

TABLE 2
Association of sRAGE and esRAGE with CHD and stroke with adjustment for other factors

Model		CHD (N events = 128)		Stroke (N events = 144)	
		HR*	P value	HR	P value
1†	sRAGE	1.57 (1.17–2.11)	0.003	0.77 (0.47–1.28)	0.31
	esRAGE	1.36 (1.04–1.77)	0.023	0.88 (0.55–1.24)	0.35
2‡	sRAGE	1.66 (1.20–2.30)	0.002	0.67 (0.39–1.15)	0.15
	esRAGE	1.43 (1.10–1.87)	0.008	0.85 (0.55–1.31)	0.46
3§	sRAGE	1.74 (1.25–2.41)	0.002	0.63 (0.37–1.09)	0.1
	esRAGE	1.45 (1.11–1.89)	0.006	0.81 (0.52–1.24)	0.33

*Effect sizes are for a doubling of sRAGE/esRAGE. †Model 1 adjusts for age, sex, and treatment group. ‡Model 2 extends model 1 by also adjusting for lipids, BMI, ethnicity, smoking, SBP, diabetes duration, and HbA_{1c}. §Model 3 further adjusts for baseline eGFR and albuminuria.

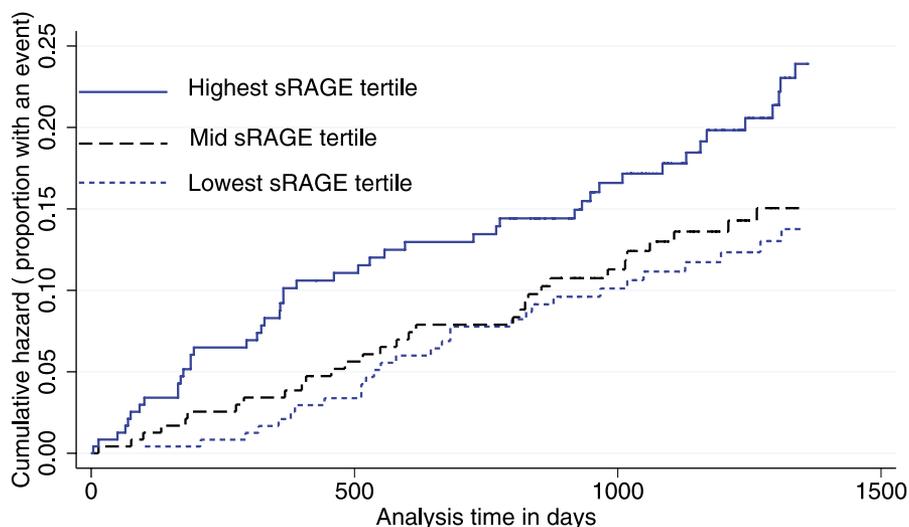


FIG. 2. CHD incidence by tertile of sRAGE adjusted for age, sex, treatment group, lipids, BMI, ethnicity, smoking, SBP, diabetes duration, HbA_{1c}, baseline eGFR, and albuminuria. (A high-quality color representation of this figure is available in the online issue.)

factors associated with baseline sRAGE and/or esRAGE, the association was independent of other factors. As shown in Table 2, model 3 adjusted for eGFR and albuminuria status, but this had very little effect on the relationship of sRAGE or esRAGE with CHD. There was no significant association between ethnicity and CHD (HR for white vs. nonwhite = 0.6; $P = 0.1$), and adjusting for ethnicity did not alter the association of esRAGE or sRAGE with CHD (model 2). Restricting the analysis to whites only showed similar effects (for example for model 1, the HR of CHD for sRAGE restricted to whites was 1.58; $P = 0.004$). There were too few nonwhites ($n = 50$) to model CVD events in these participants separately. Adjusting for BMI slightly strengthened the association of both sRAGE and esRAGE with CHD (model 2). The relationship of esRAGE to CHD was slightly weaker than the association of sRAGE with CHD ($P = 0.001$; difference in log likelihood for the two models = -2). In these models, esRAGE and sRAGE were log₂ transformed because of their skewed distribution. Thus the HRs show the effect of a doubling of the sRAGE or esRAGE level. The data were consistent with a linear relationship of sRAGE with CHD. The HR for the mid-tertile adjusted for age, BMI, and ethnicity was slightly elevated at 1.11 (0.7–1.75) and for the top tertile was 1.62 (1.06–2.5), with the P value for linear trend across tertiles being 0.025. Using a logistic regression model, the same conclusions were reached.

Also shown in Table 2, the HRs for the association of sRAGE or esRAGE with stroke were in the opposite direction to that for CHD but were not statistically significant, and the CIs are wide, consistent with the small number of stroke events ($n = 44$).

We examined the effect of atorvastatin on sRAGE. As shown in Fig. 3, sRAGE levels were very stable in both treatment arms between baseline and 1-year follow-up. Using samples from 469 people ($n = 285$ allocated placebo and 184 allocated atorvastatin) who had prerandomization and 1-year postrandomization samples available for sRAGE, there was no effect of atorvastatin (10 mg daily) on within-person change in sRAGE (β for treatment allocation = -3.5 ; 95% CI -87 to 81 , $P = 0.9$) in a regression model of follow-up RAGE adjusted for baseline sRAGE, age, and sex. Given

the lack of atorvastatin effect on sRAGE and the strong correlation between esRAGE and sRAGE at baseline, quantification of follow-up esRAGE was not considered worthwhile.

DISCUSSION

These data are novel and important in demonstrating that both higher sRAGE and higher esRAGE levels predict future CHD events in type 2 diabetes in a prospective study. Our findings add in an important way to the literature on sRAGE and vascular events in type 2 diabetes. To date, as thoroughly reviewed by Kalea et al. (2), most of the studies examining this question have been very small; few have involved adjusting for a wide range of factors, and most have been either cross-sectional studies of sRAGE with atheroma burden (13,14), prospective intima-media thickness or angiography studies (16,18), or case-control studies of existing cases of CVD (9). Perhaps not surprisingly, given this, the literature is conflicting. Of note, to our knowledge, no previous studies have measured both circulating sRAGE and esRAGE in the same type 2 diabetic subjects followed prospectively for vascular events. In the first prospective study of sRAGE and 85 incident cardiovascular events in type 1 diabetes, higher levels of sRAGE were associated with incident vascular events, as in our study. The study showed those in the middle and third tertiles of sRAGE had HRs of 1.33 and 1.78, respectively (8). Stroke was not examined separately in that study. In our analysis, although being in the mid-tertile for sRAGE was not significantly associated with CHD, considered separately, the HR for the mid-tertile was 1.11 with a significant linear trend, consistent with a continuous rather than threshold effect of sRAGE on CHD. In the previous report of sRAGE and CVD in type 1 diabetes, this continuous nature of the relationship of sRAGE with events was more apparent. We have reported the association of future CHD events with sRAGE and esRAGE measured at just a single time point. Although the correlation between sRAGE at baseline and at 1 year follow-up was very high, suggesting that this is a highly stable marker, it would nonetheless be of interest to know whether the average of

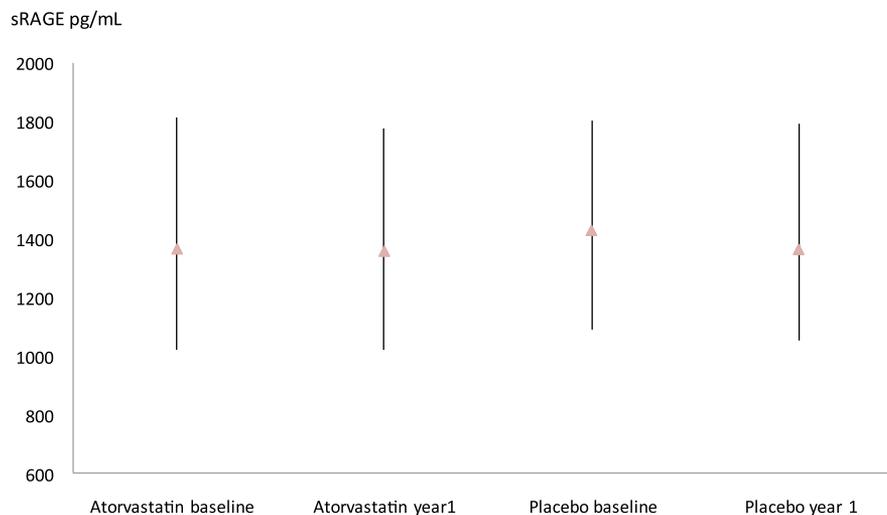


FIG. 3. Median (interquartile range) sRAGE at baseline and 1 year postrandomization by treatment group. (A high-quality color representation of this figure is available in the online issue.)

several measurements would be more strongly associated with CHD. Future studies should address how these markers vary through time in relation to progression of vascular disease.

The relationship with CHD was independent of the wide range of other factors we considered, including eGFR. There has been discussion over whether associations of sRAGE with vascular events merely reflect a secondary rise in sRAGE with declining eGFR rather than a causal relationship. In our data, adjusting for baseline eGFR did not explain the relationship with CHD. In contrast, in the recent prospective study in type 1 diabetes, ~28% of the relationship of sRAGE with vascular events was explained by renal function in regression analyses. In that study, the authors noted that even then the direction of causality cannot be deciphered unequivocally and of course animal studies are consistent with at least some effect of RAGE signaling on renal disease (19,20).

Our study yields several other important novel pieces of information about circulating sRAGE and esRAGE. First we demonstrated that circulating serum sRAGE and esRAGE levels are highly correlated in patients with diabetes. Consistent with this, they share similar associations with other risk factors. As reported in an earlier smaller study, we note that, based on comparing the concentrations of circulating esRAGE with sRAGE, most of the circulating sRAGE seems to be from cleaved wild-type RAGE rather than esRAGE (5). We also demonstrated that there are large differences in sRAGE and esRAGE levels between ethnic groups with lower sRAGE and esRAGE in those of African Caribbean origin compared with whites, with South Asians having intermediate levels. This is consistent with previous reports of whites having higher levels than those of African or Hispanic origin (21,22). We ensured that ethnicity was not confounding the relationship between sRAGE and CHD by adjusting for it in the modeling process and by confirming the association in a model restricted to whites. We found that higher BMI is associated with lower sRAGE and esRAGE, which is not what one might have expected if higher levels of each are associated with CHD. However, lower sRAGE was found associated with higher BMI in nondiabetic subjects previously (23) and was associated with metabolic syndrome (14) and some other inflammatory

states, such as rheumatoid arthritis (24). Whether these lower levels of sRAGE relate to its role as a decoy for RAGE ligands in such inflammatory states is unclear. There was little correlation of sRAGE with glycemic control. The weak inverse association of HbA_{1c} and esRAGE levels suggests that exploration of whether factors related to glycemia, possibly AGEs, alter splicing is of interest.

We confirm that those with poorer renal function have higher levels of sRAGE and esRAGE. However, it is noteworthy that relatively little interindividual variation in sRAGE or esRAGE levels is explained by the many patient characteristics we examined (<10%). This and the ethnic differences suggest strong genetic control of sRAGE and esRAGE levels, consistent with emerging literature on RAGE genetics (2). We also demonstrate in this large trial that statin therapy has little effect on sRAGE levels, supporting the importance of the development of specific RAGE inhibitors. This is in conflict with some smaller studies suggesting effects of statins (14,25).

There are many potential mechanisms through which RAGE signaling may be related to vascular disease in diabetes (1,26). For both sRAGE and esRAGE, the existing knowledge suggests possible opposing relationships of circulating levels with vascular disease. For example, circulating soluble RAGE may act as a decoy for other ligands that bind to RAGE, thereby having an anti-atherosclerotic effect; if this mechanism is dominant, then one would expect that higher sRAGE relates to fewer vascular events. However, there is also emerging evidence that proteolytic cleavage, through which the component of sRAGE that does not derive from esRAGE (so-called cleaved RAGE) is formed, is part of a regulatory process and may reflect ongoing inflammation (4,5), in which case one would expect higher levels to be associated with more vascular disease. It is also unclear whether elevated esRAGE levels would predict more or less complications. Our analysis shows that for CHD, the predominant relationship is a positive one, higher levels are associated with higher disease incidence, and suggests that given current assays, circulating cleaved RAGE may be a more useful predictor of CHD than esRAGE. Clearly if we had been able to assay cleaved RAGE separately, this would have been valuable for testing this latter point directly.

We found no significant relationship with stroke, and indeed the HR was in the opposite direction to that for CHD. However, the power for examining relationships with stroke events was low with only 44 stroke events in this study. It is possible that higher sRAGE levels may have a different relationship with disease in the coronary vasculature than in brain, perhaps reflecting increased inflammation-related processes in coronary disease but being more a marker of decreased RAGE ligand binding in brain. It is interesting that, consistent with this, a recent study examining sub-clinical stroke reported that higher sRAGE was associated with fewer subclinical strokes (21). That there could be different associations of sRAGE with disease in brain than other tissues is also plausible given that proteolysis of RAGE, which would lead to higher levels of sRAGE, has been postulated to prevent Abeta peptide transport across the blood-brain barrier and thereby β -amyloid deposition (4). In turn, there is increasing evidence that the extent of β -amyloid deposition can influence the degree of brain injury with ischemic episodes and alter vascular function in brain (27).

Of course we realize it is important to differentiate between steady-state circulating levels of wild-type sRAGE and the cell-surface expression of sRAGE. Circulating levels will presumably reflect both expression of RAGE and proteolytic cleavage of RAGE and possibly other factors, and these may differ in their importance for pathogenic effects. The relationship between circulating levels of sRAGE and expression levels of RAGE is likely to be complex (6). Many splice variants of RAGE are not secreted (6). Thus, our study of circulating serum levels focuses on sRAGE and esRAGE more from the perspective of their potential usefulness as biomarkers rather than inferring what is happening with respect to RAGE expression or binding of ligands to RAGE. A first step in demonstrating potential usefulness of biomarkers is the evidence of a prospective relationship as demonstrated here, but detailed analysis of the marginal predictive utility of sRAGE as a biomarker and its role in net reclassification improvement will require a study with many more events, perhaps achieved through meta-analysis.

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H.M.C. contributed to designing the substudy, analyzed the data, and prepared the first draft of the manuscript. D.J.B. contributed to designing this substudy and edited the manuscript. P.D. contributed to designing this substudy, edited the manuscript, and also generated the lipid data. G.H. and A.N. contributed to designing this substudy and edited the manuscript. S.L. contributed to data analysis and edited the manuscript. V.C.-M. contributed to designing this substudy, edited the manuscript, also generated the lipid data. W.B. contributed to data analysis and edited the manuscript. D.A.D. and G.M.P. contributed to designing this substudy and edited the manuscript. H.D. contributed to data analysis and edited the manuscript. K.T. edited the manuscript. J.H.F. contributed to designing this substudy and edited the manuscript.

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