Advanced Glycation End Products, Oxidation Products, and Incident Cardiovascular Events in Patients With Type 2 Diabetes

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Juraj Koska,[1] Aramesh Saremi,[1] Scott Howell,[2] Gideon Bahn,[3] Barbora De Courten,[4] Henry Ginsberg,[5] Paul J. Beisswenger,[2] and Peter D. Reaven,[1] for the VADT Investigators*

OBJECTIVE

The goal of this study was to determine whether plasma levels of advanced glycation end products (AGE) and oxidation products (OP) predict the incidence of cardiovascular disease (CVD) in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Five specific AGE (methylglyoxal hydroimidazolone, carboxymethyl lysine, carboxyethyl lysine, 3-deoxyglucosone hydroimidazolone, and glyoxal hydroimidazolone) and two OP (2-aminoadipic acid and methionine sulfoxide [MetSO]) were measured at baseline in two intensive glucose-lowering studies: *1*) a subcohort of the Veterans Affairs Diabetes Trial (VADT) (*n* = 445) and *2*) a nested case-control subgroup from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study (*n* = 271).

RESULTS

Increased levels of several AGE and OP were associated with older age, decreased kidney function, previous CVD, and longer diabetes duration, but not with hemoglobin A_{1c}. In the VADT, increased risk of incident CVD events (*n* = 107) was associated with lower MetSO after adjusting for age, race/ethnicity, sex, prior CVD event, kidney function, treatment assignment, and diabetes duration (hazard ratio [HR] 0.53; 95% CI 0.28–0.99; *P* = 0.047). Individuals with both low MetSO and high 3-deoxyglucosone hydroimidazolone concentrations were at highest risk for CVD (HR 1.70; *P* = 0.01). In the ACCORD study, those with incident CVD events (*n* = 136) had lower MetSO (by 14%; *P* = 0.007) and higher glyoxal hydroimidazolone and carboxymethyl lysine (by 18% and 15%, respectively; *P* = 0.04 for both); however, only the difference in MetSO remained significant after adjustment for prior CVD event (*P* = 0.002).

CONCLUSIONS

Lower levels of MetSO and higher levels of select AGE are associated with increased incident CVD and may help account for the limited benefit of intensive glucose lowering in type 2 diabetes.

[1]Phoenix VA Health Care System, Phoenix, AZ
[2]PreventAGE Healthcare, Lebanon, NH
[3]Hines VA Cooperative Studies Program Coordinating Center, Edward Hines Jr. VA Hospital, Hines, IL
[4]Monash Centre for Health Research and Implementation, School of Public Health and Preventive Medicine, Monash University, Clayton, Victoria, Australia
[5]Department of Medicine, Columbia University, New York, NY

Corresponding author: Juraj Koska, juraj.koska@va.gov.

Cardiovascular diseases (CVD) are the major cause of mortality in patients with type 2 diabetes (T2D). Despite a well-established role for chronic hyperglycemia in CVD risk (1), intensive glycemic control for up to 6 years at best modestly reduces the development of CVD in people with longstanding T2D (2–4). These incongruent results raise the possibility that vascular changes resulting from years of chronic hyperglycemia might lessen the effect of intensive glucose lowering in these studies.

Advanced glycation end products (AGE) and related oxidative end products (OP) are reactive intermediates of chronic hyperglycemia with proteins, lipids, and nucleic acids, resulting in long-lasting modification of these substrates (5–7). The formation of AGE and OP is intertwined; increased AGE may lead to oxidative stress (8) and, vice versa, reactive oxygen species (ROS) may facilitate AGE formation (9). Both AGE and OP are increased in diabetes and have been thought to induce, in part, the cardiovascular complications of diabetes (6,7)

In line with in vitro and preclinical evidence linking AGE and OP to atherosclerosis and CVD (reviewed by Shah and Brownlee [5]), several clinical and epidemiological studies indicate an association between various AGE and CVD events (10–19). We recently found a significant association between several plasma AGE and OP and progression of carotid intima media thickness and calcified coronary atherosclerosis in patients with T2D participating in the Veterans Affairs Diabetes Trial (VADT) (20).

In this study we demonstrate that plasma levels of AGE and OP were also associated with the risk of clinical CVD events in these same VADT participants, and we confirm these findings in a nested case-control cohort of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) lipid trial (21).

RESEARCH DESIGN AND METHODS

The samples and the data for this study came from the baseline visits from two independent studies of intensive glucose lowering in patients with T2D. The main cohort was a subset of 445 participants of the VADT (4), which was previously used to study the long-term effects (after VADT) of glucose-lowering therapy on measures of carotid and coronary atherosclerosis (22). The primary CVD outcome for the current study was the first occurrence of any one of a composite of nonfatal macrovascular events, including myocardial infarction; stroke; new or worsening congestive heart failure; surgical intervention for cardiac, cerebrovascular, or peripheral vascular disease; inoperable coronary artery disease; and amputation for ischemic gangrene (4). The confirmatory cohort was a nested case-control subgroup of 271 participants (135 cases, 136 controls) from the ACCORD lipid trial (21). The primary

outcome (cases) in the ACCORD trial was the first occurrence of nonfatal myocardial infarction or nonfatal stroke or death from cardiovascular causes (3). Cases and controls were matched by age, sex, race/ethnicity, and study treatment. All participants provided written informed consent to participate in the VADT and ACCORD studies, both of which are registered at clinicaltrials.gov (identifiers NCT00032487 and NCT00000620, respectively).

As described previously (20,23), five dicarbonyl-derived AGE—Nε-carboxymethyl lysine (CML), Nε-carboxyethyl lysine (CEL), glyoxal hydroimidazolone (G-H1), methylglyoxal hydroimidazolone (MG-H1), and 3-deoxyglucosone hydroimidazolone (3DG-H1)—and two OP, methionine sulfoxide (MetSO) and 2-aminoadipic acid (2-AAA), were measured by liquid chromatography (LC)–mass spectrometry (MS) using internal stable heavy isotope substituted standards (PreventAGE Healthcare Technology). Analysis was performed in a blinded fashion on the plasma filtrate following centrifugation through 10,000 cutoff Amicon filters. This fraction contains free AGE and OP, as well as peptides of various sizes, and the analytical method measured the free products. An Agilent model 6490 Triple Quadrupole MS system with a 1290 Rapid Resolution LC system was used to detect analytes. All AGE and OP were separated and analyzed in a single run using a single Waters X-select HSS T3 column (2.5 μm, 2.1 × 150 mm) with a mobile phase gradient of methanol and water with 0.20% heptafluorobutyric acid. Total analysis time ran 19 min. Interassay

coefficients of variation varied from 3.6% (2-AAA) to 9.6% (G-H1).

Statistical analyses were performed with the SAS statistical package version 9.4 (SAS Institute, Cary, NC). Data were $\log_{10}$ transformed if they were not normally distributed. P values <0.05 were considered statistically significant. Between-group differences were assessed with the Student t test for continuous variables and the χ^2 test for categorical outcomes. Univariate associations between the continuous variables were assessed by Spearman correlation. Mixed-model ANCOVA was used to test the differences between the groups after adjusting for subject-specific random effects and fixed effects of potential confounders. Risk of incident cardiovascular events in the VADT was analyzed by Cox proportional hazards models adjusted for treatment assignment. Additional adjustments were made for age, race/ethnicity, sex, prior CVD event, kidney function, treatment assignment, and diabetes duration. Combinations of AGE and OP predicting CVD events were selected by stepwise Cox regression, with $P < 0.2$ for entry into and $P < 0.1$ for retention in the model. Cumulative probability curves constructed using the Kaplan–Meier method compared subjects at low and high risk, as defined by selected median AGE and OP or their combinations.

RESULTS
The clinical and demographic characteristics within each subset of participants were comparable with those of the full VADT and ACCORD lipid cohorts,

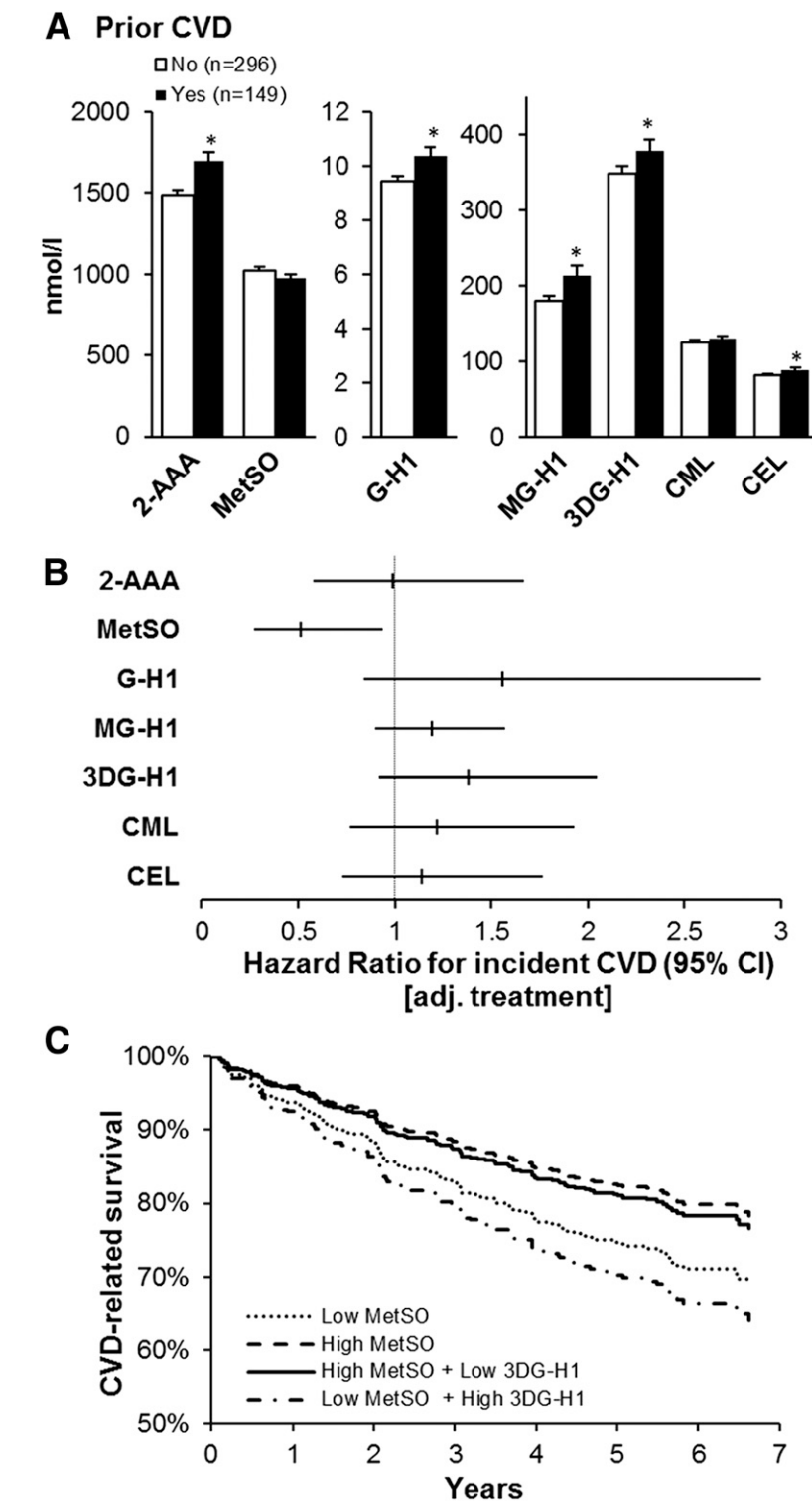### Table 1—Association of AGE and OP with clinical characteristics at baseline

Characteristics, by cohort	2-AAA	MetSO	G-H1	MG-H1	3DG-H1	CML	CEL
Age							
VADT	−0.03	0.12*	0.31§	0.23§	0.18§	0.16‡	0.26§
ACCORD	−0.18*	0.02	0.14*	0.12	0.11	0.09	0.06
BMI							
VADT	0.22§	−0.03	−0.01	0.03	0.01	0.02	0.00
ACCORD	0.01	−0.12	−0.04	−0.01	−0.004	0.07	0.08
Diabetes duration							
VADT	0.02	0.08	0.18‡	0.17‡	0.17‡	0.14†	0.13†
ACCORD	−0.11	0.00	0.11	0.12*	0.08	0.18*	0.17*
Hemoglobin A_{1c}							
VADT	0.02	−0.04	−0.02	−0.05	−0.01	0.01	−0.08
ACCORD	0.00	0.03	−0.09	−0.07	0.00	−0.09	−0.06
GFR							
VADT	−0.14†	−0.09	−0.43§	−0.29§	−0.30§	−0.35§	−0.45§
ACCORD	−0.15*	−0.11	−0.50§	−0.37*	−0.37*	−0.51*	−0.39*

Data are Spearman correlation coefficients. GFR, glomerular filtration rate (Modification of Diet in Renal Disease formula). *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$, §$P < 0.0001$.

respectively; they comprised predominantly white, overweight or obese men with a median 10-year history of diabetes and suboptimal glycemic control (Supplementary Table 1). In both subset cohorts, all AGE and 2-AAA negatively correlated with kidney function, and several of them positively correlated with age. All AGE and 2-AAA positively correlated with diabetes duration but not with hemoglobin A_{1c} (Table 1). Those with a history of CVD events before enrolling in the VADT had significantly higher 2-AAA (mean ± SD 1,699 ± 639 vs. 1,484 ± 551 nmol/L; $P = 0.0002$), GH-1 (10.4 ± 4 vs. 9.5 ± 3 nmol/L; $P = 0.003$), MG-H1 (214 ± 166 vs. 179 ± 135 nmol/L; $P = 0.01$), 3DG-H1 (379 ± 182 vs. 348 ± 177 nmol/L; $P < 0.05$), and CEL (88 ± 51 vs. 81 ± 37 nmol/L; $P = 0.03$) (Fig. 1A). However, only the significant difference in 2-AAA levels persisted after adjusting for age, race/ethnicity, sex, diabetes duration, and kidney function ($P = 0.0002$).

During the VADT, a total of 107 patients within this cohort subset developed a CVD event after a median of 2.3 years (range 0.1–6.6 years). These patients with incident CVD events were older, more likely to be non-Hispanic white, and had a history of a previous CVD event and a longer diabetes duration (Table 2). Higher plasma MetSO concentrations were associated with a lower risk of incident CVD events (Fig. 1B), and this association was independent of age, race/ethnicity, sex, prior CVD event, kidney function, treatment assignment, and diabetes duration (hazard ratio [HR] 0.53 [95% CI 0.28–0.99]; $P = 0.047$). Although a trend was seen for increased CVD risk for several AGE in the treatment-adjusted models, these did not reach statistical significance (Fig. 1B). In stepwise modeling evaluating all AGE and OP, however, both 3DG-H1 (HR 1.51 [95% CI 1.003–2.27]; $P < 0.05$) and MetSO (HR 0.45 [95% CI 0.27–0.85]; $P = 0.01$) were associated with incident CVD. The combination of both low MetSO and high 3DG-H1 further increased the risk for CVD beyond low MetSO alone (Fig. 1C). This combination remained a significant predictor of CVD after adjusting for age, race/ethnicity, sex, prior CVD event, kidney function, treatment assignment, and diabetes duration (HR 1.70 [95% CI 1.12–2.57]; $P = 0.01$).

In the ACCORD study, those with prior CVD had higher G-H1 (mean ± SD 13 ± 4 vs. 11 ± 4 nmol/L; $P < 0.0001$), 3DG-H1 (402 ± 205 vs. 330 ± 165 nmol/L; $P = 0.002$), and CML (144 ± 72 vs. 115 ± 63 nmol/L; $P = 0.0004$) (Fig. 2A); however, only the higher G-H1 remained significant ($P = 0.007$) after adjusting for age, race/ethnicity, sex, diabetes duration, and kidney function. History of CVD was also more common in those with incident CVD

Figure 1—Baseline plasma OP and AGE, and prevalent and incident CVD, in the VADT. A: OP and AGE by CVD history. Data are mean ± SE. *$P < 0.05$ between groups. B: Cox proportional HR and 95% CI for treatment-adjusted (adj.) effects of OP and AGE for incident CVD. C: Kaplan-Meier curves of CVD-related survival stratified by high and low MetSO alone or in combination with low and high 3DG-H1, respectively (both stratified by median).

Table 2—Baseline demographic and clinical characteristics by incident CVD events in the VADT and the ACCORD subcohorts included in these analyses

	VADT		ACCORD	
	CVD (*n* = 107)	No CVD (*n* = 338)	CVD (*n* = 135)	No CVD (*n* = 136)
Participants receiving intensive treatment	50	52	47	51
Age (years)	60 ± 8†	58 ± 8	64 ± 6	64 ± 6
Male sex	99	96	76	75
Non-Hispanic white	72*	60	72	68
Prior CVD	59§	25	57§	33
BMI (kg/m²)	31 ± 5	31 ± 4	32 ± 5	32 ± 5
Diabetes duration (years)	13 ± 8‡	10 ± 7	12.7 ± 7.3	10.5 ± 7
Hemoglobin A_{1c} (% [mmol/mol])	9.3 ± 1.3 (79 ± 14)	9.5 ± 1.5 (80 ± 17)	8.2 ± 0.9 (67 ± 10)	8.3 ± 1.0 (67 ± 11)
Cholesterol (mmol/L)				
Total	4.8 ± 1.5	4.7 ± 0.9	4.5 ± 0.9	4.7 ± 1.0
LDL	2.8 ± 0.8	2.7 ± 0.8	2.4 ± 1.0	2.7 ± 0.8
HDL	0.91 ± 0.21	0.95 ± 0.26	0.93 ± 0.17	0.96 ± 0.19
Triglycerides (mmol/L)	1.8 ± 0.8	1.9 ± 0.9	2.2 ± 1.1	2.4 ± 1.4
GFR (mL/min/1.73 m²)	80 ± 22	83 ± 18	86 ± 25	90 ± 21

Data are mean ± SD or percentages. Incident composite CVD events in the VADT included myocardial infarction; stroke; new or worsening congestive heart failure; surgical intervention for cardiac, cerebrovascular, or peripheral vascular disease; inoperable coronary artery disease; and amputation for ischemic gangrene. Incident composite CVD in the ACCORD included myocardial infarction, stroke, and cardiovascular death. GFR, glomerular filtration rate (Modification of Diet in Renal Disease formula). *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$, §$P < 0.0001$, independent samples *t* test or χ^2 test, as appropriate.

(i.e., cases; median time to an event 3 years [range 1–7 years]) than in controls (Table 2). Incident CVD cases had lower MetSO (1,637 ± 802 vs. 1,901 ± 801 nmol/L; *P* = 0.007) and higher G-H1 (13 ± 4 vs. 11 ± 4 nmol/L) and CML (137 ± 79 vs. 119 ± 56 nmol/L) (*P* = 0.04 both) (Fig. 2*B*); however, only the difference in MetSO remained significant after adjustment for CVD history (*P* = 0.002).

CONCLUSIONS

We found that higher levels of MetSO in both the VADT and ACCORD subset cohorts remained strongly associated with reduced incident CVD events in middle-aged and older patients with a relatively long history of T2D. Several other AGE and OP were also associated with prevalent and incident CVD. Although most AGE associations did not remain significant after adjusting for major risk factors or history of CVD, higher concentrations of 3DG-H1 in combination with lower MetSO levels further enhanced risk for incident CVD in the VADT.
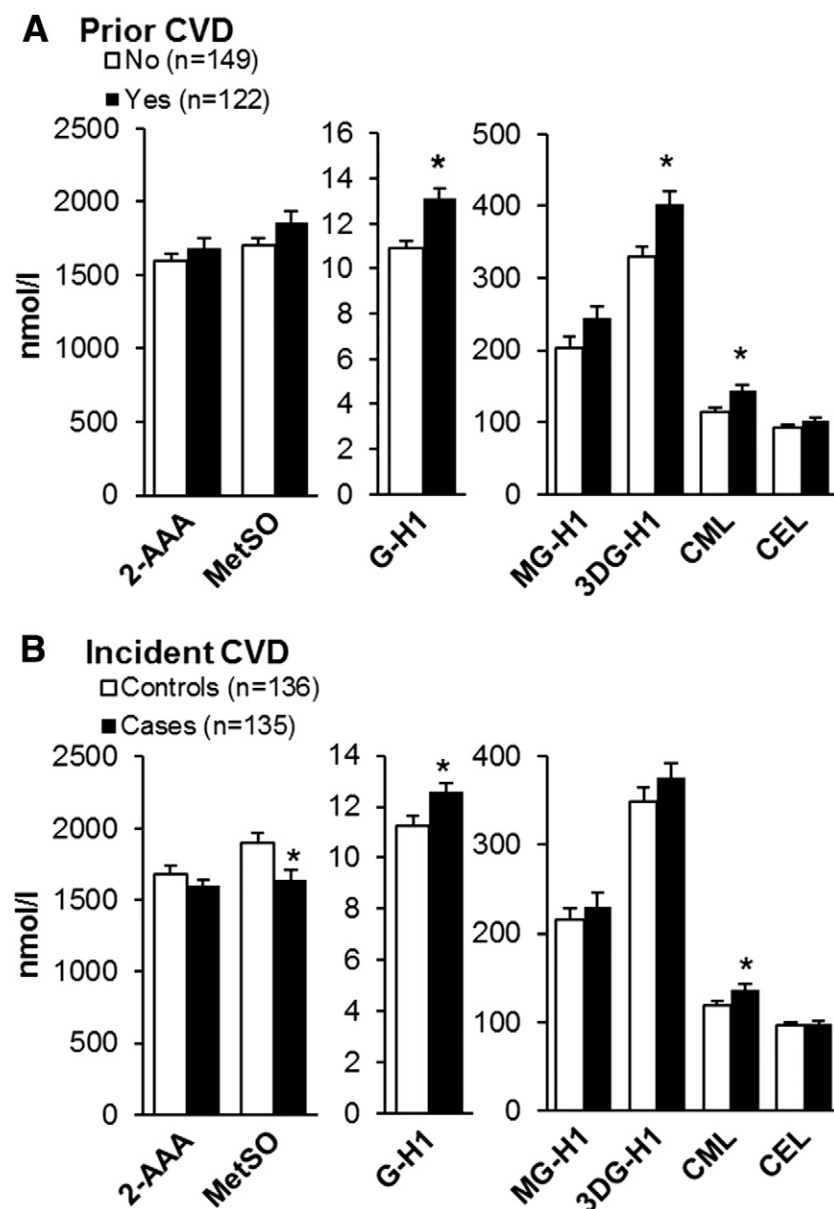
Thus, in two separate cohorts, we observed the novel finding that a significant association exists between lower MetSO and incident CVD. Consistent with these results, a recent preliminary report of type 1 diabetes from the Epidemiology of Diabetes Interventions and Complications (EDIC)/Diabetes Control and Complications Trial (DCCT) cohort showed an inverse association between plasma MetSO and incident CVD (24). Because MetSO is formed by ROS-mediated oxidation of methionine residues of multiple proteins, its inverse association with CVD events seems to contradict the notion of a deleterious effect of oxidative stress on cardiovascular health (5). However, methionine residues on protein molecules have been proposed to constitute an important antioxidant defense mechanism, as a variety of oxidants react readily with methionine to form the less reactive methionine sulfoxide (25). Surface-exposed methionine residues provide an efficient scavenger system and protect other functionally essential residues from oxidative damage. Furthermore, MetSO can be reduced back to methionine by the enzyme methionine sulfoxide reductase, providing a catalytic amplification of the antioxidant potential of each methionine residue (26). Our findings can be interpreted as showing that those with lower MetSO levels have less effective protection against oxidative stress through this pathway, resulting in greater oxidative damage to vascular cells. MetSO formation can also be an important autoregulatory component in cell signaling. For example, methionine sulfoxidation of calcium-calmodulin has been identified to contribute to downregulation of cellular metabolism and ATP utilization and, as a consequence, reduced ROS generation under stress conditions (27).

As a result of their potential to accumulate in tissue over the long term and the ability to initiate and promote vascular damage (28), AGE and select OP are considered important contributors to the development of microvascular and macrovascular complications in T2D (5). In support of this concept, several studies showed increases in various AGE or glycoxidation products in T2D and CVD (10–14). In this analysis using LC-MS in two different cohorts, plasma levels of multiple free AGE and OP adducts were indeed increased in individuals with pre-existing CVD; however, these differences seemed to be largely explained by standard CVD risk factors and demographic characteristics. In fact, only 2-AAA in the VADT and G-H1 in the ACCORD cohorts remained significantly higher in those with prevalent CVD after adjusting for these potential confounders. Our results show some similarity to a previous report by Hanssen et al. (10), who detected associations between several AGE and prevalent CVD in individuals with normal glucose metabolism, impaired glucose metabolism, or T2D, although none of these were independent of traditional CVD risk factors.

Consistent with several previous longitudinal studies that have reported associations between plasma AGE and incident CVD (15,18,19,29), we found a nonsignificant trend for an increased risk for CVD for

Figure 2—Baseline plasma AGE and OP in an ACCORD nested case subcohort by history of CVD (*A*) and incident CVD (*B*) during the study. Data are mean ± SE. *$P < 0.05$ between groups.

Consistent with the prolonged accumulation of these products with persistent hyperglycemia and oxidative stress, plasma concentrations of several AGE and OP were directly associated with duration of diabetes. The poor association between AGE and OP with hemoglobin A_{1c} also emphasizes the minor effect of short- to moderate-term glycemia changes on these adducts in the setting of advanced T2D. This may also partly explain the modest protective effect of intensive glucose lowering in patients with advanced T2D observed during both the VADT and ACCORD trial (3,4).

Our study has several potential limitations. Despite a moderate number of CVD events in both study subsets, our cohorts may not have had adequate statistical power to detect small effects of some AGE and OP. In addition, because the original VADT subcohort used in this analysis was designed to examine long-term atherosclerosis changes, it did not include individuals who died during the VADT (20). This may have modified the CVD risk associated with AGE and OP. Because age and renal function are important determinants of death and were associated with higher AGE concentrations, which are in turn associated with all-cause and CVD mortality (15,18), excluding these individuals may have underestimated the association of AGE with CVD. We believe it is important that we found similar associations of CVD with AGE and MetSO in the confirmatory ACCORD subcohort that included CVD death within its primary outcome. Further studies of the full VADT and ACCORD cohorts and their ongoing long-term observational follow-up studies will clarify these issues. Although lower glomerular filtration rate was associated with higher levels of most AGE and 2-AAA in our study, consistent with prior reports (32,33), its association with MetSO was weak and does not account for relationships between MetSO and incident CVD. Although it does not lessen the importance of the associations between AGE and CVD, in this study we could not account for dietary AGE as a source of free AGE in plasma (34,35). However, dietary AGE modestly increase free AGE in plasma, mostly during the postprandial period, and they are cleared relatively quickly via the kidneys (34,36). Thus their contribution to plasma levels was likely small after an overnight fast in our study subjects with preserved kidney function.

most AGE in the VADT. 3DG-H1 did become a significant predictor of CVD events after inclusion with MetSO in a combined model. This supports the concept that AGE may contribute to CVD; we previously observed a significant positive association between several AGE and OP and long-term progression of atherosclerosis, defined by vascular calcification or carotid intima media thickness, in this same VADT subcohort (20). In the ACCORD subcohort, G-H1 and CML were higher in those who developed incident CVD; however, this increase was partially explained by preexisting CVD. It is possible that median follow-up times of 5.6 and 4.3 years,

respectively, may be too short to detect a stronger association between higher AGE and OP and clinical CVD outcomes. In fact, the follow-up time in a previous study demonstrating a significant association between plasma AGE and incident CVD events was nearly 10 years (29).

Weak associations of plasma AGE with clinical markers of glycemic control, as observed in this study, have been noted previously in patients without diabetes (30). This is not surprising, as the majority of free AGE in the circulation originate from long-lived tissue and cellular stores in the body (31) and thus are not readily influenced by ambient glucose control.

Finally, the VADT cohort comprised largely white men; thus our results cannot be generalized to the entire population. Of note, however, some previous studies suggest the possibility of a stronger association of selected AGE and incident CVD in women compared with men (15,19).

In conclusion, our results indicate that lower levels of MetSO and higher levels of select AGE are associated with incident cardiovascular events over 3–7 years of follow-up in patients with long-standing T2D. This may help explain the limited benefit of intensive glucose-lowering therapy in these moderate-duration studies.

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