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Plasma Advanced Glycation End Products Are Associated With Incident Cardiovascular Events in Individuals With Type 2 Diabetes: A Case-Cohort Study With a Median Follow-up of 10 Years (EPIC-NL)

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Experimental data suggest a role for advanced glycation end products (AGEs) in cardiovascular disease (CVD), particularly in type 2 diabetes (T2DM). However, epidemiological evidence of an association between high plasma AGEs and increased cardiovascular risk remains inconclusive. Therefore, in a case-cohort study comprising 134 cardiovascular case subjects and a random subcohort of 218 individuals (including 65 cardiovascular case subjects), all with T2DM and nested in the European Prospective Investigation into Cancer and Nutrition in the Netherlands (EPIC-NL) study, plasma levels of protein-bound N_{ε}-(carboxymethyl)lysine, N_{ε}-(carboxyethyl)lysine, and pentosidine were measured with liquid chromatography. AGEs were $\log_e$-transformed, combined in a *z*-score, and the association with incident cardiovascular events was analyzed with Cox proportional hazard regression, adapted for case-cohort design (Prentice method). After multivariable adjustment (sex, age, cohort status, diabetes duration, total cholesterol to HDL-cholesterol ratio, smoking, systolic blood pressure, BMI, blood pressure–, cholesterol- and glucose-lowering treatment, prior cardiovascular events, and triglycerides), higher plasma AGE *z*-scores were associated with higher risk of incident cardiovascular events in individuals without prior cardiovascular events (hazard ratio 1.31 [95% CI: 1.06–1.61]). A similar trend was observed in individuals with prior cardiovascular events (1.37 [0.63–2.98]). In conclusion, high plasma AGEs were associated with incident cardiovascular events in individuals with T2DM. These results underline the potential importance of AGEs in development of CVD.

Experimental studies link advanced glycation end products (AGEs), a heterogeneous family of sugar-modified proteins, to cardiovascular disease (CVD) and other complications of diabetes (1–3). Several AGE-lowering compounds have been shown to reduce atherosclerosis in animal models (4,5), demonstrating that AGEs are no innocent bystanders and are of importance for the development of CVD.

The formation of AGEs is a complex and heterogeneous process, yielding numerous different AGE adducts, derived from several distinct metabolic processes. AGEs are formed as a result of the Maillard reaction (i.e., a slow

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and passive process of months in which sugars react with protein residues on long-lived proteins), leading to cross-linking AGEs such as pentosidine. Pentosidine has been associated with vascular stiffening (6). In addition, glucose, and also lipids, can undergo auto-oxidation, leading to AGEs such as N_{ε}-(carboxymethyl)lysine (CML) (7), a well-known ligand for the receptor for AGEs, which plays a pivotal role in inflammatory signaling (8). Furthermore, AGEs are formed during high glycolytic activity, leading to intracellular accumulation of the AGE precursor methylglyoxal (9), which reacts with arginine and lysine to form AGEs like 5-hydro-5-methylimidazolone and N_{ε}-(carboxyethyl)lysine (CEL). Intracellular formation of AGEs especially takes place in cells that do not regulate their glucose uptake, such as endothelial cells, and induces oxidative stress and endothelial dysfunction (10,11).

In addition to previous animal work, it has been shown that AGEs accumulate in human atherosclerotic plaques (12,13). Furthermore, levels of AGEs are higher in rupture-prone plaques than in stable plaques and may explain the association between inflammation and growth of a necrotic core in advanced atherosclerosis (14). These findings indicate that AGEs may indeed play an important role in the development of cardiovascular events in humans. However, epidemiological studies using plasma measurements of AGEs, which are more feasible than plaque measurements, have generated inconsistent associations with incident cardiovascular events (15–20). These results may be conflicting because they were based on semiquantitative ELISA-based techniques to measure AGEs (measuring mostly one AGE only) instead of specific liquid chromatography–based techniques and did not take potential confounding factors (consistently) into account. To address this issue, we investigated associations between plasma AGE levels, measured with liquid chromatography, and prior cardiovascular events and found that AGEs were not associated with prior cardiovascular events, after adjustment for potential confounders, in a large cross-sectional study containing individuals with and without type 2 diabetes (T2DM) (21). However, in a prospective study among individuals with type 1 diabetes, we did find associations between higher specific plasma AGEs and higher risk of incident cardiovascular events, after adjustment for confounders (22).

Therefore, the aim of the current study was to address whether plasma AGEs, measured with state-of-the-art liquid chromatography, are associated with incident cardiovascular events in individuals with T2DM. To this end, we measured the major specific protein-bound AGEs CML, CEL, and pentosidine in a case-cohort study nested in the prospective European Prospective Investigation into Cancer and Nutrition in the Netherlands (EPIC-NL) study.

RESEARCH DESIGN AND METHODS

Study Population

The EPIC-NL cohort is the Dutch contribution to the EPIC study and consists of the Prospect-EPIC and MORGEN-EPIC cohorts (23). In brief, the Prospect-EPIC study includes 17,357 women 49–70 years of age living in Utrecht, the Netherlands, and vicinity who participated in the nationwide Dutch breast cancer screening program between 1993 and 1997. The MORGEN-EPIC cohort consists of 22,654 men and women 21–64 years of age selected from random samples of the Dutch population in three different towns. Participants were recruited in both studies from 1993 to 1997.

The study population consisted of all participants with confirmed T2DM at baseline (n = 536). Three sources of ascertainment of diabetes were used: self-report, hospital discharge diagnoses, and urinary strip test (in the Prospect part of the cohort only). Ascertained cases of diabetes were verified against medical and pharmacy records. Details of the ascertainment sources and verification procedures have previously been described (24).

All participants provided written informed consent before study inclusion.

The study complies with the Declaration of Helsinki and was approved by the institutional board of the University Medical Center Utrecht (Prospect) and the Medical Ethical Committee of The Netherlands Organization for Applied Scientific Research Nutrition and Food Research (MORGEN).

Outcome Assessment

Follow-up data on incident cardiovascular events were obtained from the Dutch Centre for Health Care Information, which holds a standardized computerized register of hospital discharge diagnoses. All diagnoses were coded according to the ICD-9. Follow-up was complete until 1 January 2008. The database was linked to the cohort on the basis of birth date, sex, postal code, and general practitioner with a validated probabilistic method (25). Information on vital status was obtained through linkage with the municipal registries. Causes of death were collected from Statistics Netherlands and coded according to the ICD-10. Primary outcome for the present analysis was incident cardiovascular events (fatal and nonfatal). A cardiovascular event was defined as coronary heart disease (CHD), peripheral arterial disease (PAD), congestive heart failure (CHF), and stroke (CVD, 410–414, 427.5, 428, 415.1, 443.9, 430–438, 440–442, 444, 798.1, 798.2, and 798.9; CHD, 410–414, 427.5, 798.1, 798.2, and 798.9; PAD, 440–444; heart failure, 428; and stroke, 430–434 and 436). These end points included both fatal and nonfatal cases. If a participant developed multiple cardiovascular events, the first event was taken into account in the analyses of total incident cardiovascular events. Due to limited statistical power, fatal events were analyzed separately only for total cardiovascular events.

Case-Cohort Sampling

Case-cohort sampling was performed to reduce costs and preserve valuable biological material. All incident cardiovascular cases (n = 134) from the baseline patients with T2DM (n = 536) were included in this analysis. A random sample of 218 participants was selected to serve as a subcohort in

the case-cohort design. Due to the random selection, 65 participants within the subcohort developed an incident cardiovascular event and overlapped between the subcohort and the cases, an inherent feature of case-cohort studies. A total of 153 participants served as non–case subjects inside the subcohort, and 69 cardiovascular case subjects were outside the subcohort.

AGE Measurements

Protein-bound plasma AGEs CML, CEL, and lysine were measured with ultraperformance liquid chromatography tandem mass spectrometry, as described previously (21). Protein-bound plasma pentosidine was measured using high-performance liquid chromatography, as described previously (26). In the present analyses for CML, CEL, pentosidine, and lysine, intra-run and inter-run variations were 7.7, 5.0, 6.2, and 6.7% and 3.3, 4.7, 6.2, and 5.2%, respectively. All plasma AGEs were adjusted for lysine as a marker for total plasma protein.

Other Measurements

The general questionnaire addressed demographic characteristics and presence of and risk factors for chronic diseases. Smoking was categorized into current, past, and never smoker. During the baseline physical examination screening, systolic and diastolic blood pressure measurements were performed twice (from which the mean was taken) in the supine position on the right arm using a Boso Oscillomat (Bosch & Son, Jungingen, Germany) (Prospect-EPIC) or on the left arm and using a random zero sphygmomanometer (MORGEN-EPIC). Height and weight were measured by trained staff, and BMI was calculated as weight in kilograms divided by squared height in meters. Time since diabetes diagnosis was calculated by subtracting the age of diagnosis from the age at baseline examination. Glycated hemoglobin (HbA_{1c}), plasma cholesterol, creatinine, and triglycerides were measured (23), and the estimated glomerular filtration rate (eGFR) was calculated (27), as described previously. All measurements were performed according to standard operating procedures.

Statistical Analyses

All analyses were performed using R 2.15.1 for Windows. Missing values on covariates were replaced using single imputation to reduce bias and increase statistical power.

We $\log_e$-normalized all plasma AGEs to reduce the potential influence of outliers and to obtain a normal distribution, needed for the calculation of *z*-scores. The AGE score was calculated by averaging (and subsequently standardizing) the *z*-scores of CEL, CML, and pentosidine. To increase statistical power while reducing the risk of chance findings, we performed all primary analyses with AGE score as the exposure.

We investigated the baseline characteristics of the subcohort across tertiles of the AGE score. Categorical variables were expressed as frequencies. Means and SDs were presented for normally distributed variables, whereas non–normally distributed variables were reported as medians and interquartile ranges. Linear trend was tested with ANOVA or χ^2, as appropriate. Next, in a combined sample (n = 287) of the subcohort and case subjects outside the subcohort, we used linear regression analyses to investigate the cross-sectional associations of age, sex, $\log_e$ diabetes duration, systolic blood pressure, smoking, total to HDL-cholesterol ratio, BMI, blood pressure–, glucose-, and cholesterol-lowering treatment, prior cardiovascular events, $\log_e$ triglycerides, HbA_{1c}, and eGFR with plasma AGEs. These covariates were adjusted for each other and for cohort and presented as standardized betas (βs) with 95% CIs.

Cox proportional hazard regression models were used to investigate the associations between plasma AGEs with incident cardiovascular events, accounting for the case-cohort design using the method of Prentice (28). In brief, all subcohort members (case subjects and non–case subjects) were weighted equally, and cases outside the subcohort were not weighted before failure. At time of event, these case subjects had the same weight as the subcohort members and only then contributed to the risk sets. Results from a case-cohort study closely resemble analyses on a full cohort (29). All models were adjusted for sex, age, and cohort (model 1). Furthermore, we additionally adjusted for traditional cardiovascular risk factors (i.e., duration of diabetes, smoking, systolic blood pressure, total to HDL-cholesterol ratio, prior cardiovascular events, triglycerides, BMI, and cholesterol-, glucose-, and blood pressure–lowering treatment; model 2). As decreased kidney function and poor glycemic control may be potential sources of higher plasma AGEs, we wanted to investigate factors in the causal chain, which we analyzed by adjusting for those variables in a separate step (model 3). Cox proportional hazard estimates are presented as hazard ratios (HRs) and 95% CIs. We stratified our main analyses by prior cardiovascular events. By adding interaction terms to model 3, we investigated whether associations were modified by prior cardiovascular events (prior cardiovascular events × AGE) or cohort status (cohort × AGE). $P < 0.10$ was considered statistically significant for the tests of interaction. Due to limited statistical power, analyses on fatal cardiovascular events and on CHD, stroke, PAD, and CHF were analyzed in the entire population.

RESULTS

Median follow-up time was 10.7 (6.0–12.6) years, and 21.6% of the incident cardiovascular events were fatal. Some individuals (n = 19) developed more than one event, resulting in 150 documented events (80 CHD, 19 stroke, 30 PAD, and 21 CHF). Table 1 shows the baseline characteristics across tertiles of the AGE score. Higher age, lower BMI and eGFR, and no lipid-lowering treatment were significantly associated with higher plasma AGEs in unadjusted analyses. Supplementary Table 1 shows the baseline characteristics for the subcohort and cardiovascular case subjects.

Determinants of Plasma AGEs

Next, we investigated which covariates were independently associated with plasma AGEs. Lower eGFR (β −0.37 [95% CI −0.49 to −0.24]) was associated with a higher AGE score, but HbA_{1c} was not associated with plasma AGEs (0.02 [−0.09 to 0.13]). Glucose-lowering treatment was associated with a lower AGE score (−0.39 [−0.70 to −0.08]). This was also the case for cholesterol- and blood pressure–lowering treatment, but these associations were not statistically significant (Table 2). We also found that a lower BMI was associated with a higher AGE score (−0.23 [−0.34 to −0.12]). Age was also inversely associated with the AGE score (−0.14 [−0.28 to 0.00]) (Table 2). We found no association between prior cardiovascular events and the AGE score. When we analyzed the plasma AGEs separately, we overall found similar results, except for CEL, which was not associated with BMI and glucose-lowering treatment (Table 2). These results were similar when we excluded the case subjects outside the subcohort from analyses (data not shown).

Plasma AGEs and Incident CVD

After adjustment for sex, age, and cohort, we found a nonsignificant association between plasma AGEs and incident cardiovascular events in individuals without (HR 1.16 [95% CI 0.93–1.44]) and with prior cardiovascular events (1.13 [0.73–1.74]). After additional adjustment for diabetes duration, total cholesterol to HDL-cholesterol ratio, smoking, systolic blood pressure, prior cardiovascular events, triglycerides, BMI, glucose-, lipid-, and blood pressure–lowering treatment (Table 3, model 2), a higher plasma AGE score was statistically significantly associated with higher risk of incident cardiovascular events in individuals without prior cardiovascular events (1.31 [1.06–1.61]). A similar trend was observed in individuals with prior cardiovascular events (1.37 [0.63–2.98]). Additional adjustment for eGFR and HbA_{1c} did not affect these results in individuals without prior cardiovascular events (1.40 [1.10–1.78]). Although in individuals with prior cardiovascular events, additional adjustment for HbA_{1c} and eGFR seemed to attenuate the association (0.64 [0.30–1.35]), there was no statistical interaction between individuals with and without prior cardiovascular events (Table 3, model 3).

When we analyzed the AGEs separately, we found overall similar associations for CML (without prior cardiovascular events, HR 1.47 [95% CI 1.10–1.97]; with prior cardiovascular events, 0.98 [0.53–1.82]) and CEL (without prior cardiovascular events, 1.39 [1.12–1.72]; with prior cardiovascular events, 0.74 [0.44–1.23]), while pentosidine was not associated with incident CVD events (without prior cardiovascular events, 1.15 [0.89–1.49]; with prior cardiovascular events, 0.95 [0.59–1.54]) (Table 3, model 3). The associations between AGEs and incident cardiovascular events in patients without prior cardiovascular events were largely similar to those for the entire population, and, although point estimates differed considerably between individuals with and without prior cardiovascular events in stratified analyses, particularly in model 3, we did not find any statistical interactions between individuals with and without prior cardiovascular events (Table 3).

Therefore, we investigated in the entire population which confounder changed the association between the plasma AGE score and cardiovascular events in model 1 compared with models 2 and 3 (Table 3). When we omitted BMI from model 3, we no longer found an association between the plasma AGE score and incident cardiovascular events (HR 1.13 [95% CI 0.89–1.44]). We found similar results for CML and pentosidine when we omitted BMI from the model, while CEL remained significant (1.31 [1.04–1.67]). In line, when we adjusted for waist circumference instead of BMI, we found near-identical results (1.29 [1.01–1.66]).

Associations Between Plasma AGEs and Specific Cardiovascular Outcomes

Furthermore, we analyzed the associations between plasma AGEs and the major cardiovascular outcome CHD as well as the less frequent outcomes (fatal cardiovascular events, stroke, PAD, and CHF) (Supplementary Table 1, model 3). Higher plasma AGEs were associated with a significantly higher risk of stroke (HR 2.06 [95% CI 1.13–3.75]) and a borderline significantly higher risk of CHD (1.32 [0.96–1.80]). Higher plasma AGEs were also associated with higher risk of fatal cardiovascular events (1.16 [0.85–1.62]) and CHF (1.73 [0.77–3.91]), but these associations were not significant. In contrast, although nonsignificant, higher plasma AGEs were associated with lower risk of PAD (0.76 [0.43–1.34]). This association was strongest for pentosidine (0.67 [0.40–1.13]) (Supplementary Table 1, model 3).

DISCUSSION

The main finding of this study was that, after adjustment for confounding factors, higher plasma levels of protein-bound AGEs were associated with higher risk of incident cardiovascular events in individuals with T2DM. Overall, the associations were independent of HbA_{1c} and eGFR. When we analyzed the AGEs separately, we found similar associations with incident cardiovascular events for CML and CEL, but not pentosidine. Results were largely consistent for the subtypes of cardiovascular events, except for PAD, which was inversely associated with the plasma level of AGEs, and pentosidine in particular.

In line with our previous report (21), we again observed no independent association between plasma AGEs and prior cardiovascular events in cross-sectional analyses. In contrast, and in line with our previous work in type 1 diabetes (22), we found that higher plasma AGE levels were associated with increased risk of incident cardiovascular events, particularly after adjustment for confounders (mainly BMI). The lack of associations with prior cardiovascular events may be due to limitations of cross-sectional analyses, such as inclusion of case subjects only with favorable outcome at baseline. In addition, AGE levels may be susceptible to interventions to treat CVD and may not

Table 1—Baseline characteristics across tertiles of the plasma AGE score in the subcohort (n = 218)

	Lowest tertile	Middle tertile	Highest tertile	P_{trend}
Participants (n)	73	72	73	—
Female (%)	76.7	80.6	87.7	0.09
Age (years)	56.7 ± 7.2	58.1 ± 6.2	59.1 ± 6.4	0.03
Diabetes duration (years)	3.9 (1.5–9.9)	5.3 (2.3–10.0)	6.1 (2.9–12.9)	1.00
HbA_{1c} (%)	7.9 ± 1.6	8.2 ± 1.7	8.1 ± 1.6	0.44
HbA_{1c} (mmol/mol)	63.0 ± 17.5	66.0 ± 18.6	65.0 ± 17.5	0.44
Glucose-lowering treatment (%)	91.8	90.3	84.9	0.19
SBP (mmHg)	141.4 ± 20.1	141.0 ± 20.3	142.7 ± 22.2	0.71
DBP (mmHg)	83.4 ± 11.5	81.5 ± 9.6	81.4 ± 9.3	0.24
Use of blood pressure–lowering drugs (%)	37.0	34.7	43.8	0.40
Prior cardiovascular events (%)	11.0	19.4	11.0	1.00
Current smokers (%)	30.1	13.9	23.3	0.33
BMI (kg/m^2)	31.5 ± 4.8	29.7 ± 4.7	27.1 ± 4.0	<0.01
Waist (cm)	102.3 ± 11.6	97.4 ± 12.1	92.1 ± 11.2	<0.01
Serum creatinine (μmol/L)	58.3 ± 12.6	58.7 ± 15.4	67.3 ± 24.8	<0.01
eGFR ($mL/min/1.73\ m^2$)	99.7 ± 13.1	97.0 ± 14.9	89.1 ± 18.0	<0.01
Total cholesterol (mmol/L)	5.3 ± 1.3	5.2 ± 1.0	5.1 ± 1.2	0.26
HDL cholesterol (mmol/L)	1.0 ± 0.2	1.0 ± 0.3	1.1 ± 0.3	0.12
Triglycerides (mmol/L)	2.3 (1.6–3.4)	1.9 (1.5–3.0)	1.9 (1.0–2.6)	1.00
Use of lipid-lowering drugs (%)	8.2	2.8	1.4	0.04
Protein-bound plasma AGEs				
CML (nmol/mmol lysine)	52.1 (46.1–59.6)	63.7 (57.8–73.1)	80.4 (72.1–91.7)	<0.01
CEL (nmol/mmol lysine)	23.5 (20.7–26.7)	29.2 (26.0–31.9)	32.1 (27.3–38.4)	<0.01
Pentosidine (nmol/mmol lysine)	0.5 (0.4–0.6)	0.6 (0.5–0.8)	1.0 (0.8–1.3)	<0.01

Data are presented as frequencies (%), means ± SD, or medians (interquartile range). Linear trend was tested with ANOVA or χ^2, as appropriate. Skewed variables (diabetes duration, triglycerides, and the AGEs) were $\log_e$-transformed prior to analyses. DBP, diastolic blood pressure; SBP, systolic blood pressure.

remain elevated after a cardiovascular event has taken place. This is conceivable because several lipid-lowering (30), glucose-lowering (31), and antihypertensive compounds (32) have been shown to have AGE-lowering properties, and we indeed found that less glucose-lowering treatment was associated with higher plasma AGEs, with similar trends for blood pressure– and lipid-lowering treatment (Table 2).

Moreover, obesity seems to have a large influence on AGE levels. We and others showed an inverse association between BMI and AGEs (CML in particular) (15,33), and we have postulated trapping of AGEs in tissues in obesity (8). This is further substantiated by the current study, as we indeed show that in individuals with T2DM, BMI was inversely associated with plasma AGEs. In fact, only when we adjusted the association of the AGE score with incident cardiovascular events for BMI, we found significant associations. In line with this, adjusting for waist, another marker for adiposity, yielded identical results. Furthermore, we observed that the direction of the association between age and plasma AGE levels inverted after adjustment for confounders (Table 1 vs. Table 2). When we added the confounders separately to the model, we discovered this was mainly driven by confounding due to eGFR (data not shown). When we stratified the analyses for the presence of prior cardiovascular events, we found, overall, largely similar results between individuals with and without prior cardiovascular events. In model 3, however, we could not detect any significant association in individuals with prior cardiovascular events. It is unlikely that this is due to a genuine difference between individuals with and without prior cardiovascular events. It is much more likely that this was due to limited statistical power in individuals with prior cardiovascular events, as from the 50 individuals with prior cardiovascular events, 39 developed a cardiovascular event, and none of the interaction terms were statistically significant. Nonetheless, investigation of the associations between plasma AGEs and incident cardiovascular events in a larger cohort comprising individuals with established CVD would be needed to fully address this question.

With this study, we show that a prospective design and adjustment for confounders have a large impact on the associations between plasma AGEs and incident cardiovascular events (Table 3, model 1 vs. models 2 and 3) (21). Therefore, inconsistencies in previous literature may be largely explained by inconsistent adjustment for confounding, as associations with incident cardiovascular events differed greatly with or without

Table 2—Multivariable βs and 95% CIs for the cross-sectional associations between covariates and plasma AGEs

	AGE score		CML		CEL		Pentosidine	
Independent variable	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Age (years)	−0.14	−0.28 to 0.00	−0.12	−0.26 to 0.02	−0.13	−0.28 to 0.02	−0.06	−0.20 to 0.09
Sex (male is reference)	−0.13	−0.49 to 0.24	−0.28	−0.63 to 0.08	0.16	−0.23 to 0.56	−0.17	−0.53 to 0.19
Diabetes duration (years)	0.07	−0.04 to 0.18	0.05	−0.06 to 0.15	0.15	−0.03 to 0.27	−0.04	−0.15 to 0.07
Systolic blood pressure	0.07	−0.04 to 0.19	0.11	0.00 to 0.22	0.04	−0.08 to 0.17	0.01	−0.10 to 0.13
Smoking (nonsmoking is reference)	−0.07	−0.21 to 0.07	−0.20	−0.33 to 0.06	0.11	−0.04 to 0.26	−0.06	−0.20 to 0.08
Total to HDL-cholesterol ratio	0.07	−0.06 to 0.19	0.06	−0.06 to 0.18	0.00	−0.13 to 0.14	0.09	−0.04 to 0.21
BMI	−0.23	−0.34 to −0.12	−0.28	−0.39 to −0.17	0.03	−0.10 to 0.15	−0.26	−0.37 to 0.14
BP-lowering treatment (no is reference)	−0.15	−0.38 to 0.09	−0.20	−0.42 to 0.03	−0.10	−0.35 to 0.15	−0.04	−0.27 to 0.20
Cholesterol-lowering treatment (no is reference)	−0.44	−0.98 to 0.10	−0.23	−0.74 to 0.29	−0.75	−1.32 to −0.17	−0.01	−0.54 to 0.53
Prior cardiovascular events (no is reference)	−0.18	−0.47 to 0.12	−0.18	−0.46 to 0.10	−0.16	−0.48 to 0.16	−0.06	−0.35 to 0.24
Triglycerides (mmol/L)	−0.08	−0.21 to 0.05	−0.20	−0.33 to −0.08	0.13	−0.01 to 0.26	−0.13	−0.23 to −0.03
Glucose-lowering treatment (no is reference)	−0.39	−0.70 to −0.08	−0.31	−0.61 to −0.01	0.00	−0.33 to 0.33	−0.57	−0.88 to −0.26
HbA_{1c} (%)	0.02	−0.09 to 0.13	0.04	−0.06 to 0.15	0.00	−0.12 to 0.12	0.00	−0.11 to 0.12
eGFR (mL/min/1.73 m^2)	−0.37	−0.49 to −0.24	−0.34	−0.46 to −0.22	−0.15	−0.28 to −0.01	−0.34	−0.46 to 0.22

Data were analyzed using linear regression analyses. Diabetes duration and triglycerides were $\log_e$-transformed prior to analyses. βs are expressed per 1 SD increase of independent variable, as 1 SD change of AGE score, or $\log_e$-transformed AGE. All independent variables were adjusted for each other and for cohort status.

Table 3—Associations between plasma AGEs and incident cardiovascular events in the entire population and stratified according to prior cardiovascular events

		Incident cardiovascular events			
	Model	Entire population (n = 287; 134 case subjects)	No prior events (n = 237; 95 case subjects)	Prior events (n = 50; 39 case subjects)	$P_{interaction}$
AGE score	1	1.11 (0.91–1.35)	1.16 (0.93–1.44)	1.13 (0.73–1.74)	—
	2	1.30 (1.02–1.65)	1.31 (1.06–1.61)	1.37 (0.63–2.98)	—
	3	1.33 (1.03–1.71)	1.40 (1.10–1.78)	0.64 (0.30–1.35)	0.865
CML	1	1.00 (0.81–1.24)	1.06 (0.71–1.56)	1.07 (0.75–1.52)	—
	2	1.31 (0.99–1.73)	1.35 (1.05–1.73)	1.14 (0.35–3.70)	—
	3	1.34 (0.99–1.82)	1.47 (1.10–1.97)	0.98 (0.53–1.82)	0.950
CEL	1	1.21 (0.99–1.49)	1.26 (1.00–1.58)	1.07 (0.68–1.69)	—
	2	1.31 (1.04–1.65)	1.31 (1.07–1.60)	1.09 (0.59–2.00)	—
	3	1.34 (1.06–1.70)	1.39 (1.12–1.72)	0.74 (0.44–1.23)	0.933
Pentosidine	1	1.04 (0.84–1.28)	1.07 (0.67–1.72)	1.06 (0.75–1.51)	—
	2	1.03 (0.80–1.33)	1.12 (0.91–1.39)	1.24 (0.70–2.17)	—
	3	1.02 (0.78–1.33)	1.15 (0.89–1.49)	0.95 (0.59–1.54)	0.450

Data were analyzed using Cox regression analyses. HR is expressed per SD increase of $\log_e$-transformed AGE. Model 1: adjusted for age, sex, and cohort; model 2: model 1 + adjustment for diabetes duration, total cholesterol to HDL-cholesterol ratio, smoking, systolic blood pressure, prior cardiovascular events, triglycerides, BMI, and glucose-, lipid-, and blood pressure–lowering treatment; and model 3: model 2 + eGFR and HbA_{1c}.

adjustment for these confounding factors. Other studies focusing on plasma AGEs and incident cardiovascular events found associations between higher plasma CML and higher risk of incident cardiovascular events in older individuals (15,16), while no associations were found in individuals with T2DM (17). In hemodialysis patients, even associations between higher CML levels and lower risk of incident cardiovascular events have been described (18). Finally, Kilhovd et al. (19,20) have found that higher serum levels of AGEs and 5-hydro-5-methylimidazolone were associated with higher risk of incident cardiovascular events, but only in nondiabetic women. The majority of previous studies have used stepwise regression models (17,18) or selected covariates based on significance in univariable analyses (15,16). These analyses are less appropriate for etiological analyses, as they are not hypothesis driven. Thus, selection of the covariates may be based more on statistical power of the study than on underlying biology and subsequently yield inconsistent models. Indeed, most studies adjusted for BMI (15,16,19,20), but others did not (17,18), and no study adjusted for glucose-, blood pressure–, and cholesterol-lowering medication.

We consistently found that CEL in particular was strongly associated with incident cardiovascular events. This finding is in line with the concept that methylglyoxal and its derived AGEs, such as CEL, play the most important role in AGE accumulation (1) and development of diabetes complications (34,35). However, this finding should be interpreted with some caution, as for CML and pentosidine, we are likely to underestimate associations with outcome due to residual negative confounding from obesity, as BMI (or waist circumference) does not completely capture adiposity. Furthermore, all associations between plasma AGEs and incident cardiovascular events were independent of HbA_{1c} and eGFR. This is in line with the current concept that AGE accumulation is a complex and heterogeneous process, in which factors such as lipid peroxidation and oxidative stress may play a large role (8), and is not simply dependent on hyperglycemia or decreased renal clearance in diabetes.

Interestingly, higher AGEs (particularly crosslinking AGE pentosidine) were associated with lower risk of PAD (which included abdominal aneurysm–related disease). Although we acknowledge that our current study was underpowered to properly address associations with abdominal aneurysms only, this is a very interesting finding. Crosslinking by AGEs may in fact strengthen the vessel wall, reducing aneurysm formation. These results are in line with a previous cross-sectional study focusing on CML and abdominal aneurysms (36) and may perhaps explain the well-known paradox that individuals with diabetes have an increased risk of CVD, but a lower risk of progression of abdominal aneurysms (37). An adequately powered study should investigate associations between plasma AGEs and development of abdominal aneurysms and PAD as separate end points to address this issue.

The current study shows that associations between plasma AGEs and incident cardiovascular events differ considerably from individuals with type 1 diabetes (22). In particular, we found a major confounding effect by obesity, but not HbA_{1c}, and we found no strong positive associations between plasma pentosidine and incident cardiovascular events. Besides their role in CVD, AGEs have been linked to development of microvascular complications of diabetes (1), but interestingly, some plasma AGEs have been linked to higher, while other have been linked to lower risk of microvascular complications (38). As the associations between plasma AGEs and microvascular

complications in T2DM are largely unknown, this should be addressed in a future study.

To the best of our knowledge, our study is the first cohort study investigating associations between plasma AGEs and incident cardiovascular events in T2DM using a large number of cardiovascular case subjects and state-of-the-art liquid chromatography for assessment of several specific protein-bound AGEs in plasma. Nevertheless, our study has some limitations. As the current study population consists of Caucasians who all had T2DM, our findings may not apply to the general population without diabetes. In addition, although we measured three major specific AGEs, we could not measure the full spectrum of AGEs. Also, as most AGEs are produced intracellularly and/or in tissues, it is most likely that not all AGEs reach the circulation, and we may therefore underestimate the actual strength of the association between AGE accumulation and incident cardiovascular events by measuring plasma AGEs. To this end, AGE measurements that better reflect accumulation of tissue AGEs are needed (such as plasma methylglyoxal levels or AGEs in circulating cells). Furthermore, our study was underpowered to investigate associations between plasma AGEs and specific CVD outcomes, in particular stroke, PAD, and CHF. Therefore, although overall largely consistent, these results should be seen as hypothesis generating. This is particularly true for the protective associations with PAD, which deserve further research. Furthermore, more severe and acute manifestations of CVD, like myocardial infarction or stroke, are easier to diagnose than less severe or chronic manifestations of CVD, such as angina pectoris, CHF, PAD, or transient ischemic attack. Also, severe cases are more likely to be treated in the hospital and thus registered in the Hospital Discharge Registries. Therefore, for outcomes with many minor events, we may underestimate the associations, as misclassification is more likely than for outcomes with more severe events. This may have resulted in weaker associations (39), which may not necessarily reflect a weaker biological association.

In conclusion, higher levels of plasma AGEs are associated with higher risk of incident cardiovascular events in individuals with T2DM. This study further underlines the importance of AGEs in development of CVD. In addition, future studies are needed to investigate whether AGE measurements in plasma, circulating cells, or urine could be developed into biomarkers to eventually improve risk prediction of CVD, particularly in individuals with diabetes.

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