Higher Plasma Levels of Advanced Glycation End Products Are Associated With Incident Cardiovascular Disease and All-Cause Mortality in Type 1 Diabetes

A 12-year follow-up study

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Diabetes is characterized by hyperglycemia and associated with increased cardiovascular disease (CVD) risk (1). Advanced glycation end products (AGEs) may link hyperglycemia to the development of vascular complications in diabetes (2). AGEs affect cells in three general ways: 1) cellular functions can be altered when intracellular proteins are modified by AGEs; 2) modification of extracellular matrix proteins results in abnormal interactions between these proteins; and 3) circulating AGEs can bind to AGE receptors, which could induce receptor-mediated production of reactive oxygen species and activate transcription factor nuclear factor-κB, thereby leading to deleterious changes in cellular processes (2).

Several studies so far have examined the associations between various plasma AGEs and microvascular and macrovascular complications in subjects with (3–12) or without (6–8,13–15) diabetes, most of which were confined to specific study populations (i.e., in elderly, patients with end-stage renal failure, patients with heart failure, or women only) (6,7,13,14) and/or had a cross-sectional design (3,5,10–12). Among the few prospective studies, most have reported positive associations between plasma AGEs and (CVD) mortality in patients with (8,9) or without (6,7,14,15) type 2 diabetes. However, so far, no prospective studies have investigated the associations between plasma AGEs and incident cardiovascular complications in patients with type 1 diabetes. In addition, the mechanisms through which AGEs could lead to the development of CVD in these individuals are unclear. Endothelial (2) and renal dysfunction (16), low-grade inflammation (2,14), and arterial stiffness (2) could constitute such mechanisms.

In view of these considerations, we have investigated, in a 12-year prospective...
follow-up study 1) whether plasma levels of $N^e$-(carboxyethyl)lysine (CEL), $N^e$-(carboxymethyl)lysine (CML), and pentosidine were associated with incident fatal and nonfatal CVD as well as all-cause mortality in individuals with type 1 diabetes and 2) whether the extent to which any such associations were explained (i.e., mediated) by markers of endothelial and renal dysfunction, low-grade inflammation, and arterial stiffness.

**RESEARCH DESIGN AND METHODS**

**Study population and design**

In 1993, 199 patients with type 1 diabetes and diabetic nephropathy defined according to clinical criteria (i.e., persistent macroalbuminuria (>300 mg/24 h) in at least two out of three previous consecutive 24-h urine collections, in the presence of diabetic retinopathy, and in the absence of other kidney or urinary tract disease) and 192 with persistent normoalbuminuria (i.e., urinary excretion rate <30 mg/24 h) were recruited from the outpatient clinic at Steno Diabetes Center for a prospective observational follow-up study. Details of the inclusion criteria and selection procedures have been described elsewhere (17). The study was approved by the local ethics committee, in accordance with the Helsinki Declaration, and all patients gave their informed written consent.

**Baseline investigations**

**Biomarkers.** Analyses of the biomarkers were done on frozen (~80°C) samples and were performed at a central laboratory by C.G.S.

The AGEs CML, CEL, and pentosidine were measured as described (18,19) with minor modifications. In brief, to measure CEL, CML, and pentosidine, 50 μL plasma was mixed with 100 μL water in a 10-mL glass tube with a Teflon-lined screw-cap. To prevent formation of AGEs from early glycation products during sample preparation, plasma samples were reduced by 500 μL sodium borohydride borate buffer (200 mmol/L, pH 9.2) before precipitation. This mixture was allowed to stand for 2 h at room temperature. Proteins were then precipitated by addition of 2 mL 20% trichloroacetic acid and centrifuged for 10 min (4°C) at 4,500 × g. The supernatant was carefully removed by aspiration with a Pasteur pipette. The protein pellet was washed once by adding 2 mL 5% trichloroacetic acid followed by centrifugation and removal of the supernatant. The samples were hydrolyzed by adding 1,000 μL 6 N HCl and incubated for 18 h at 110°C.

After hydrolysis, 100 μL of these samples were evaporated to dryness at 80°C under a stream of nitrogen gas and reconstituted in 200 μL 0.5 mmol/L tridecafluoroheptanoic acid for the CEL and CML measurements. Five microliters of this solution were injected on the ultra-performance liquid chromatography tandem mass spectrometry. Liquid chromatography was performed at 30°C using an Acquity UPLC BEH C18, 1.7 μm, 2.1 × 100 mm column (Waters, Milford, MA), and the Micromass Quattro Premier XE Tandem Mass Spectrometer (Waters) was used in the multiple reaction monitoring mode in the electrospray ionization–positive mode. The intra- and interassay coefficients of variation (CVs) were 7.3% and 2.0% for CEL and 3.5% and 3.6% for CML, respectively.

For the pentosidine measurement, 900 μL of the hydrolyzed samples were evaporated to dryness at 80°C under a stream of nitrogen gas and reconstituted in 200 μL 25 mmol/L citric acid. This solution was centrifuged for 10 min (4°C) at 4,500 × g, and 10 mL of this solution were injected on the high-performance liquid chromatography system. Detection was carried out using a Jasco type 821-FP spectrophotometer (Jasco Benelux, Maarssen, the Netherlands) set at an excitation and emission wavelength of 325 and 385 nm, respectively. The intra- and interassay CVs were 2.7% and 2.8% for pentosidine, respectively.

High-sensitivity C-reactive protein (hs-CRP) and secreted phospholipase A2 (sPLA2) were determined by enzyme-linked immunosorbent assays (ELISA) as described previously (20). Commercially available ELISA kits were used for measurements of plasma soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), and interleukin-6 (IL-6) (QuantiKine High Sensitivity; R&D Systems, Oxon, U.K.). The intra- and interassay CVs of these immunoassays were <8%.

**Other baseline assessments.** All investigations were performed in the morning after an overnight fast. No antihypertensive medication was ever prescribed in 24% of patients with nephropathy and 88% of the normoalbuminuric patients. All of the remaining patients were asked to stop their antihypertensive and diuretic treatment 8 days before the examination.

Arterial blood pressure was measured twice with an appropriate cuff size following at least a 10-min rest in the supine position. Mean arterial pressure (MAP) was calculated as [systolic blood pressure + (2 * diastolic blood pressure)]/3. Pulse pressure was calculated by subtracting the diastolic from the systolic blood pressure and used as a marker of arterial stiffness (21). BMI was calculated by dividing weight by height squared. Urinary albumin excretion (UAE) was measured by an enzyme immunoassay from 24-h urine collections. Serum creatinine concentration was assessed by a kinetic Jaffé method. In all patients glomerular filtration rate (eGFR) was estimated according to the short Modification of Diet in Renal Disease equation (MDRD) = 186 · [serum creatinine (mg/dL)]$^{-1.154}$ · [age]$^{-0.203}$ · [0.742 if patient is female]. Patients were interviewed using the World Health Organization cardiovascular questionnaire. Individuals were categorized into three groups according to their smoking status as never, former, or current smokers.

**Follow-up and study end points**

All patients were followed up to the last visit at Steno Diabetes Center until September 1, 2006 or until death (n = 82) or emigration (n = 3). All patients were traced through the national register during autumn 2006. If a patient had died before September 1, 2006, the date of death was recorded and information on the primary cause of death was obtained from the death certificate, which was reviewed by two independent observers. Additional available information from necropsy reports was also included. All deaths were classified as cardiovascular unless an unequivocal noncardiovascular cause was established. In all patients alive at the end of follow-up, nonfatal cardiovascular events were retrieved from their patient files from Steno Diabetes Center and/or other hospital records. The primary study outcome was a combined end point of fatal and nonfatal cardiovascular disease (i.e., myocardial infarction, percutaneous coronary intervention, coronary bypass grafting, amputation because of ischemia, vascular surgery for peripheral atherosclerotic disease, and stroke), and the secondary outcome was all-cause mortality.

**Statistical analyses**

All analyses were performed with the Statistical Package for Social Sciences (SPSS) version 15.0 for Windows (SPSS, Chicago, IL).
Variables with a skewed distribution, i.e., triglycerides, CRP, IL-6, sPLA2, UAE, and pentosidine, were log-transformed before further analyses. Comparisons of baseline characteristics between groups were performed with Student t or χ² tests, as appropriate. To investigate the association between CEL, CML, Ln-pentosidine, and an AGEs score (calculated by averaging the z-scores of CEL, CML, and Ln-pentosidine) on the one hand and incident CVD as well as all-cause mortality on the other, we used Cox proportional hazards regression models. These analyses were adjusted, first, for sex, age, duration of diabetes, case-control status, and A1C; second, for other cardiovascular risk factors (i.e., BMI, smoking status, total cholesterol, and MAP); and third, for the use of renin-angiotensin-aldosterone system (RAAS) inhibitors and/or other antihypertensive treatment. This model was then further adjusted for markers of renal dysfunction (i.e., eGFR_mdrd, and Ln-UAE), low-grade inflammation (expressed as a total score computed by averaging the z-scores of Ln-IL-6, Ln-CRP, sICAM-1, and Ln-sPLA2), endothelial dysfunction (expressed as a total score computed by averaging the z-scores of sVCAM-1 and sICAM-1), and arterial stiffness (i.e., pulse pressure); these markers were entered one at a time to ascertain the extent to which any such marker could attenuate (i.e., explain) the strength of the association between the AGEs and each of these outcomes.

We used linear regression analyses to investigate the cross-sectional associations between CEL, CML, Ln-pentosidine, and AGEs score on the one hand and markers of renal dysfunction, low-grade inflammation, endothelial dysfunction, and arterial stiffness on the other. Results of these analyses are expressed in standardized regression coefficients to allow comparison of the strength of the association between the AGEs and each of these variables.

Finally, we investigated whether the associations listed above differed between patients with normoalbuminuria and those with nephropathy by adding interaction terms to our models, the significance of which was judged on the basis of a P value <0.1 for the interaction term. We found no such interactions, and, therefore, all results are presented for the two groups combined.

**RESULTS**—Of the 391 patients included in this study, we excluded 17 (1 with nephropathy and 16 with normoalbuminuria) in whom follow-up data were not obtained, 4 with missing data on baseline biomarkers levels (1 patient with nephropathy and 3 patients with normoalbuminuria), 7 with end-stage renal failure, and 24 with prior CVD at baseline (21 patients with nephropathy and 3 with normoalbuminuria). Results reported here and thus refer to 339 patients (170 with persistent normoalbuminuria and 169 with nephropathy at baseline).

During the course of follow-up (median 12.3 years [interquartile range 7.6–12.5]), 82 individuals (24.2%) died; 85 (25.1%) suffered a fatal (n = 48) and/or nonfatal (n = 35) CVD event. Individuals with incident CVD events or who had died at follow-up had, at baseline, higher levels of AGEs and a more adverse atherosclerotic risk profile as illustrated by the more unfavorable levels of traditional risk factors, markers of endothelial dysfunction, low-grade inflammation, and pulse pressure (Table 1).

After adjustments for age, sex, duration of diabetes, A1C, case-control status, other risk factors, and the use of medication, the incidence of fatal and nonfatal CVD increased with a HR of 1.30 (1.03–1.66) per 1 SD increase in baseline levels of AGEs score (Table 2, model 3). The HRs for incident fatal and nonfatal CVD per 1 SD increase in baseline CEL, CML, and Ln-pentosidine were comparable: HR = 1.24 (1.01–1.52), HR = 1.22 (0.96–1.56), and HR = 1.29 (1.03–1.60), respectively (Supplementary Table 1, model 3). Similar results were found for the associations between the AGEs and incident all-cause mortality: HR = 1.22 (1.00–1.49), HR = 1.17 (0.91–1.50), and HR = 1.28 (1.03–1.59), respectively (Table 2; Supplementary Table 2 for analyses for each AGE separately).

If we excluded from the analyses the CVD cases to which an unequivocal CVD-related cause of death could not be ascribed, our results did not change: HR = 1.31 (1.02–1.70) after adjustments for age, sex, duration of diabetes, A1C, case-control status, other risk factors, and the use of medication.

The associations between AGEs and CVD and all-cause mortality were comparable between patients with normoalbuminuria and nephropathy at baseline: HR = 1.60 (95% CI 0.68–3.80) and HR = 1.35 (1.05–1.73) for CVD and HR = 1.32 (0.48–3.63) and 1.37 (1.06–1.77) for all-cause mortality, respectively. It should be noted that the wider CIs around the estimates in the normoalbuminuria group were most likely because of the relatively low number of events (21 CVDs and 18 deaths in this group).

Adjustment for markers of renal dysfunction (model 4a), low-grade inflammation (model 4b), endothelial dysfunction (model 4c), and arterial stiffness (model 4d) did not attenuate the associations between the AGEs and the study end points, despite the adverse associations between plasma AGEs and most of these pathophysiological mechanisms (Table 3; Supplementary Table 3 for the associations presented for each AGE separately).

Because the two markers (Ln-UAE and eGFR) of renal dysfunction were interrelated (standardized β = −0.52, 95% CI −0.61 to −0.43) and because both markers individually were inversely associated with AGEs (Table 3), although Ln-UAE was positively and eGFR was inversely associated with incident CVD, adjustments for renal dysfunction were performed by adjustments for both baseline Ln-UAE and eGFR to ensure the most appropriate correction for renal dysfunction (Table 3, model 4a).

**Additional analyses**

Patients were asked to stop their antihypertensive and diuretic treatment 8 days before the examination. However, 27% and 4% of patients in the nephropathy and normoalbuminuria groups, respectively, had taken antihypertensive medication on the day of examination. The associations between AGEs and study outcomes did not appreciably differ between those who did or did not withhold their medication at baseline examination, nor did they change after additional adjustment for medication withdrawal status (data not shown).

**CONCLUSIONS**—The main findings of this study were that in patients with type 1 diabetes higher baseline plasma levels of AGEs are associated with incident fatal and nonfatal CVD as well as all-cause mortality, independently of traditional cardiovascular risk factors, but also of markers of renal and endothelial dysfunction, low-grade inflammation, and arterial stiffness. AGEs may thus constitute a specific target for treatment to prevent the excess CVD and mortality observed in patients with type 1 diabetes.
Our results are in agreement with studies that have shown positive associations between plasma levels of AGEs or Amadori products, specifically non-CML AGE (11), pentosidine (3) and Amadori-albumin (22), and microvascular complications, but these studies were limited by their cross-sectional designs (3,11,22) and/or were confined to small study populations (3,11). This is the first prospective study that has examined the associations between plasma CEL, CML, and pentosidine and incident fatal and nonfatal CVD as well as all-cause mortality in a large sample of patients with type 1 diabetes and that has also addressed the potential pathophysiological mechanisms that may explain the associations observed. Our findings are also in agreement with two previous studies, which have related higher skin autofluorescence (23), as a potential marker for tissue AGEs accumulation, and AGEs levels in skin collagen (24) with incident coronary heart disease and microvascular complications in patients with type 1 diabetes, respectively. Whether and to what extent skin autofluorescence is associated with plasma AGEs levels still needs to be investigated.

We investigated three out of many different AGEs, all of which were associated with incident fatal and nonfatal CVD to a similar extent. Based on their molecular characteristics one could hypothesize that each AGE could exert unfavorable effects on cellular functions through (partially) different pathways. However, CEL (as a putative marker of intracellular glycation), CML (as a potential ligand of RAGE), and pentosidine (as one of the cross-linking effects, if any, of intracellular protein glycation, formation of cross-links in the extracellular matrix, and the
AGE-RAGE axis on increased CVD risk attributable to AGEs in type 1 diabetes.

Although some minor differences were found in the adverse associations between each of the AGEs on the one hand and markers of renal and endothelial dysfunction, low-grade inflammation, and pulse pressure on the other, overall, none of these pathophysiological mechanisms investigated explained the positive associations between the AGEs and incident fatal and nonfatal CVD as well as all-cause mortality. We cannot discard the possibility that the use of a selection of markers representing renal and endothelial dysfunction, low-grade inflammation, and arterial stiffness may have led to an underestimation of their mediating effects in those associations, however. Furthermore, other mechanisms (e.g., oxidative stress) not investigated herein could also play a role in the link between AGEs and CVD. It is noteworthy that markers of renal dysfunction, specifically UAE (but not eGFR), and of arterial stiffness, i.e., pulse pressure, did not attenuate the associations between AGEs and incident fatal and nonfatal CVD as well as all-cause mortality, but were associated with incident fatal and nonfatal CVD independently of AGEs and other risk factors (HR = 2.19 [1.17–4.10] and HR = 1.53 [1.15–2.02], respectively). These findings thus suggest that all three mechanisms may need specific monitoring/treatment to decrease excess CVD in diabetes.

There are limitations to our study. First, samples for analyses of AGEs and other biomarkers were taken at baseline only, which impedes evaluation of the impact of changes in these variables on cardiovascular outcome. Second, potential misclassification of nonspecific mortality as CVD-related mortality may have introduced nondifferential biases, in which case the estimates reported herein may have been underestimated. However, we cannot discard the possibility that our results may have been affected by possible differential underreporting of nonfatal CVD. Third, we measured plasma levels of three specific AGEs, and it is not clear whether these are representative of the total pool of AGEs. In addition, AGEs accumulate in tissue and cellular concentrations of AGEs are higher than plasma AGE levels (25). Further studies in which both plasma and tissue levels of AGEs are measured are needed to clarify the relation between the AGEs levels in these two compartments and their specific association to CVD. Fourth, we, like most of other studies conducted in humans (4–9,11–13), did not normalize the plasma AGE levels for an amino acid concentration, which may not enable direct comparison (of absolute values) with some other studies (3,5,10,12,14,15). Nevertheless, we do not think normalization for an amino acid concentration is likely to affect the associations between plasma AGEs and study end points in our present study, because in preliminary analyses (in a different cohort) we observed very strong correlations (r > 0.9) between normalized and non-normalized values of AGEs and comparable associations of normalized and non-normalized plasma AGEs levels with outcome (data not shown).

In conclusion, higher plasma levels of AGEs at baseline are associated with incident fatal and nonfatal CVD as well as

| Table 2—Associations between baseline plasma AGEs and incident primary and secondary end points (n = 339) |
|---------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Model                          | HR       | 95% CI    | P value   | HR       | 95% CI    | P value   |
| 1                              | 1.33     | 1.05–1.69 | 0.118     | 1.34     | 1.06–1.69 | 0.013     |
| 2                              | 1.36     | 1.08–1.73 | 0.011     | 1.34     | 1.06–1.69 | 0.013     |
| 3a                             | 1.35     | 1.07–1.71 | 0.013     | 1.36     | 1.07–1.72 | 0.011     |
| 3b                             | 1.31     | 1.03–1.66 | 0.026     | 1.25     | 0.99–1.59 | 0.060     |
| 3                            | 1.30     | 1.03–1.66 | 0.029     | 1.27     | 1.00–1.62 | 0.047     |
| 4a                             | 1.31     | 0.99–1.73 | 0.057     | 1.27     | 0.95–1.68 | 0.103     |
| 4b                             | 1.26     | 1.00–1.61 | 0.055     | 1.24     | 0.98–1.58 | 0.076     |
| 4c                             | 1.31     | 1.00–1.62 | 0.028     | 1.26     | 1.00–1.59 | 0.051     |
| 4d                             | 1.30     | 1.02–1.66 | 0.036     | 1.28     | 1.01–1.64 | 0.046     |

Primary end point was a combined end point of fatal and nonfatal CVD, and the secondary end point was all-cause mortality. During the course of follow-up, 82 individuals died; 85 suffered a fatal (n = 48) and/or nonfatal (n = 53) CVD event. Model 1 is adjusted for age, sex, A1C, case-control status, and duration of diabetes; model 2: model 1 + BMI, MAP, smoking status, and total cholesterol; model 3a: model 2 + RAAS inhibitors; model 3b: model 2 + other antihypertensive agents; model 3: model 2 + RAAS inhibitors and other antihypertensive agents; model 4a: model 3 + eGFR by abbreviated modification of diet in renal disease equation and Ln-UAE rate; model 4b: model 3 + low-grade inflammation score; model 4c: model 3 + endothelial dysfunction score; model 4d: model 3 + pulse pressure.

β, standardized regression coefficient indicates change in dependent variable (in SD) per 1 SD increase in baseline AGEs score. Model 1, adjusted for age, sex, duration of diabetes, case-control status, and A1C; model 2, model 1 + BMI, smoking status, MAP, total cholesterol, use of RAAS inhibitors, other antihypertensive treatment, and continuation of medication at baseline examination.

| Table 3—Associations between plasma AGEs and potential mechanisms linking AGEs to incident CVD and all-cause mortality (n = 339) |
|---------------------------------|-----------|-----------|-----------|
| Dependent variable              | β         | 95% CI    | P value   |
| Baseline eGFR<sub>MDRD</sub>    | −0.32     | −0.42 to 0.23 | <0.001 |
| Model 1                         | 0.29      | −0.39 to −0.20 | <0.001 |
| Model 2                         | −0.07     | −0.12 to −0.02 | 0.008 |
| Ln-UAE rate                     | −0.04     | −0.09 to 0.00 | 0.071 |
| Model 1                         | 0.06      | −0.02 to 0.14 | 0.123 |
| Model 2                         | 0.09      | 0.01–0.17 | 0.036 |
| Inflammatory score              | 0.13      | 0.03–0.22 | 0.008 |
| Model 1                         | 0.13      | 0.03–0.23 | 0.009 |
| Model 2                         | 0.03      | −0.08 to 0.14 | 0.591 |
| Endothelial dysfunction score   | 0.03      | −0.07 to 0.12 | 0.615 |
| Model 1                         | 0.03      | −0.08 to 0.14 | 0.591 |
| Model 2                         | 0.03      | −0.07 to 0.12 | 0.615 |
all-cause mortality in type 1 diabetes independent of other risk factors and may thus constitute a pathway, which explains, at least in part, the increased CVD risk attributable to type 1 diabetes in these patients.

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