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# Skin Advanced Glycation End Products Glucosepane and Methylglyoxal Hydroimidazolone Are Independently Associated With Long-term Microvascular Complication Progression of Type 1 Diabetes



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Six skin collagen advanced glycation end products (AGEs) originally measured near to the time of the Diabetes Control and Complications Trial (DCCT) close-out in 1993 may contribute to the “metabolic memory” phenomenon reported in the follow-up Epidemiology of Diabetes Interventions and Complications (EDIC) study. We have now investigated whether the addition of four originally unavailable AGEs (i.e., glucosepane [GSPNE], hydroimidazolones of methylglyoxal [MG-H1] and glyoxal, and carboxyethyl-lysine) improves associations with incident retinopathy, nephropathy, and neuropathy events during 13–17 years after DCCT. The complete 10-AGE panel is associated with three-step Early Treatment of Diabetic Retinopathy Study scale worsening of retinopathy ( $P \leq 0.002$ ), independent of either mean DCCT or EDIC study A1C level. GSPNE and fructose-lysine (furosine [FUR]) correlate with retinopathy progression, independently of A1C level. The complete

panel also correlates with microalbuminuria ( $P = 0.008$ ) and FUR with nephropathy independently of A1C level ( $P \leq 0.02$ ). Neuropathy correlates with the complete panel despite adjustment for A1C level ( $P \leq 0.005$ ). MG-H1 and FUR are dominant, independent of A1C level ( $P < 0.0001$ ), whereas A1C loses significance after adjustment for the AGEs. Overall, the added set of four AGEs enhances the association of the original panel with progression risk of retinopathy and neuropathy ( $P < 0.04$ ) but not nephropathy, while GSPNE and MG-H1 emerge as the principal new risk factors. Skin AGEs are robust long-term markers of microvascular disease progression, emphasizing the importance of early and sustained implementation of intensive therapy.

A large body of epidemiological evidence and animal experimentation underlay the hypothesis that

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hyperglycemia is a causative factor in the development of microvascular and neuropathic complications in diabetes (1). The demonstration by the Diabetes Control and Complications Trial (DCCT) that intensive treatment, which lowered the mean blood glucose level by  $\sim 75$  mg/dL and the mean A1C level by  $\sim 2.0\%$  (21.9 mmol/mol), compared with conventional treatment, sharply and significantly reduced the development and progression of these complications (2), confirmed this hypothesis. Nonetheless, the mechanistic connection of hyperglycemia to complications appears complex, containing multiple strands, even if glycemic exposure is primary (3). One path from hyperglycemia to complications is the formation of advanced glycation end products (AGEs), which can affect protein structure and function both directly and by cross-linking (4).

We have previously demonstrated cross-sectional associations between a panel of four AGEs and two solubility abnormalities in collagen from a skin biopsy sample obtained prior to DCCT closeout and the presence of retinopathy, nephropathy, and neuropathy in DCCT subjects (5). These associations included the early glycation product fructose-lysine (FL) (fructosamine) measured in acid hydrolysate as furosine (FUR), pentosidine, and carboxymethyl-lysine (CML). Furthermore, we subsequently demonstrated that these AGE abnormalities, especially FUR and CML, were highly correlated with the progression of retinopathy and nephropathy over the ensuing 10 years in the Epidemiology of Diabetes Interventions and Complications (EDIC) observational study of the DCCT cohort of patients (6).

Using a simultaneously obtained skin biopsy sample near DCCT closeout, which was stored at  $-80^{\circ}\text{C}$  awaiting the discovery of AGEs not available in 1993, we have now tested the hypothesis that the previously not available AGEs glucosepane (GSPNE) (7), carboxyethyl-lysine (CEL) (8), and hydroimidazolones of glyoxal (G-H1) and methylglyoxal (MG-H1) (9) determined by liquid chromatography tandem mass spectrometry, as described by Ahmed and Thornalley (10), are correlated with complication risk progression compared with the original set. In that regard, we recently reported the association between these markers and the concurrent progression of diabetes complications during the DCCT (7), which revealed that GSPNE was a particularly robust indicator of past cumulative glycemia that was strongly associated with the presence of nephropathic, retinopathic, and neuropathic outcomes at the time of the biopsy (7). The current study was consequently undertaken to determine whether GSPNE or any of the added AGEs were also associated with the long-term prospective risk of retinopathy, nephropathy, and neuropathy progression, individually or in combination with the originally measured AGEs and solubility markers during the 17 years of follow-up of this cohort during the EDIC study.

## RESEARCH DESIGN AND METHODS

### Subjects

Two hundred sixteen participants in the DCCT study from eight clinics volunteered for a skin biopsy, as

previously described (5), 1–2 years prior to DCCT closeout. One hundred twenty-three participants were from the primary cohort (1–5 years diabetes duration and no retinopathy seen on fundus photography), and 93 were from the secondary cohort (1–15 years diabetes duration and at least one retinal microaneurysm seen on fundus photography). Retinopathy, nephropathy, and neuropathy data at 10–13 years of the EDIC follow-up were available in 196 of those participants originally biopsied. During the early EDIC study period, mean A1C levels of the DCCT intensive and conventional treatment groups began to converge and became indistinguishable at  $\sim 7.9\%$  by 5 years into the EDIC study (11,12).

### Outcomes

#### Retinopathy

During the EDIC study, retinopathy was assessed by standardized seven-field fundus photography in one-quarter of the cohort each year and in the entire cohort at EDIC study years 4 and 10. All photographs were graded centrally, with graders masked to the therapy assignment, using the final Early Treatment Diabetic Retinopathy Study (ETDRS) grading scale (13) and DCCT methods (14). The progression of retinopathy during the EDIC study was defined as a three-step or greater worsening at any time from the DCCT closeout up to EDIC study year 16 on the final ETDRS scale of retinopathy severity (13) or initial scatter photocoagulation therapy during this interval, among those free of scatter laser in DCCT.

#### Nephropathy

Albumin excretion rate (AER) (15) was measured every 2 years in 1,399 participants during the EDIC study. Progression of nephropathy was defined as the initial development of microalbuminuria (AER  $\geq 40$  mg/24 h,  $n = 50$ ), dialysis ( $n = 3$ ), or kidney transplantation ( $n = 3$ ) at any time between the DCCT closeout and the EDIC study year 15 or 16, among those participants free of microalbuminuria in the DCCT. Since microalbuminuria is known to regress (16), sustained microalbuminuria (AER  $\geq 30$  mg/24 h) at two or more consecutive visits was used in a sensitivity analysis (Supplementary Tables 1–3) to verify the results of a single AER  $\geq 40$  mg/24 h.

#### Neuropathy

Clinical and nerve conduction studies performed during the DCCT were repeated in the EDIC study during years 13–14 (17). Likewise, cardiovascular autonomic neuropathy assessment performed in the DCCT was reassessed in the EDIC study, first during EDIC study years 13–14 (18) and repeated in years 16–17. Progression of neuropathy was defined as the initial development of confirmed clinical neuropathy or cardiac autonomic neuropathy in the EDIC study, among those free of both complications in the DCCT. Confirmed clinical neuropathy was defined as the presence of both definite clinical neuropathy (the presence

**Table 1—Clinical characteristics of DCCT participants at DCCT baseline and closeout**

	Primary cohort			Secondary cohort		
	Intensive (N = 65)	Conventional (N = 58)	P value	Intensive (N = 57)	Conventional (N = 36)	P value
<b>DCCT baseline</b>						
Age (years)	28 ± 6	26 ± 7	0.016	30 ± 7	30 ± 6	
Duration (months)	31 ± 19	27 ± 14		108 ± 51	90 ± 43	
A1C						
%	8.8 ± 1.9	9.1 ± 1.9		8.9 ± 1.5	8.3 ± 1.2	0.022
mmol/mol	73 ± 20.8	76 ± 20.8		74 ± 16.2	67 ± 13.1	
Mean blood glucose (mg/dL)	221 ± 83	245 ± 85		237 ± 76	214 ± 72	
Triglycerides (mg/dL)	79 ± 66	65 ± 23		97 ± 48	86 ± 56	
Cholesterol (mg/dL)	182 ± 33	174 ± 32		181 ± 36	176 ± 37	
Retinopathy (%)						
10/10: none	100	100		0	0	
20/≤20: microaneurysms only	0	0		58	61	
30/≤30: mild NPDR	0	0		23	25	
45/≤45: moderate NPDR	0	0		19	14	
AER >40 mg/24 h (%)	1.5	0.0		10.5	8.3	
Confirmed clinical neuropathy (%)	6.2	1.7		17.9	11.1	
SBP (mmHg)	116 ± 10	114 ± 11		115 ± 12	120 ± 12	0.042
DBP (mmHg)	73 ± 9	72 ± 9		73 ± 8	76 ± 10	
<b>DCCT closeout</b>						
Age (years)	34 ± 6	31 ± 7	0.013	36 ± 7	36 ± 6	
Duration (months)	101 ± 31	94 ± 23		189 ± 55	169 ± 46	
DCCT mean A1C						
%	7.1 ± 0.8	9.5 ± 1.4	<0.001	7.1 ± 0.8	8.8 ± 1.5	<0.001
mmol/mol	54 ± 8.7	80 ± 15.3	<0.001	54 ± 8.7	73 ± 16.4	<0.001
Mean blood glucose (mg/dL)	149 ± 48	239 ± 76	<0.001	152 ± 46	215 ± 83	<0.001
Triglycerides (mg/dL)	72 ± 35	76 ± 34		91 ± 57	78 ± 36	
Cholesterol (mg/dL)	175 ± 31	178 ± 33		181 ± 33	178 ± 33	
Retinopathy (%)			0.005			
10/10: none	58	36		4	3	
20/≤20: microaneurysms only	37	41		30	36	
30/≤30: mild NPDR	2	19		44	36	
45/≤45: moderate NPDR	3	3		23	25	
AER >40 mg/24 h	3.1	10.3		14.0	11.1	
Confirmed clinical neuropathy (%)	2.3	4.8		12.7	17.1	
SBP (mmHg)	115 ± 9	115 ± 11		118 ± 9	121 ± 14	
DBP (mmHg)	75 ± 9	74 ± 8		77 ± 8	77 ± 11	

Data are mean ± SD, unless otherwise indicated. DBP, diastolic blood pressure; NPDR, nonproliferative diabetic retinopathy; SBP, systolic blood pressure.

of signs and symptoms consistent with distal symmetrical polyneuropathy based on examination by a board-certified neurologist) and abnormal nerve conduction (one or more abnormal attributes in at least two anatomically distinct nerves among the sural, peroneal, or median nerves) (17). Cardiac autonomic neuropathy was defined as either an R-R interval variation of <15 or an R-R interval variation between 15 and 19.9 in combination with a Valsalva ratio of ≤1.5 or a decrease of >10 mmHg in diastolic blood pressure upon standing (17).

### Biomedical Evaluations

Demographics were assessed by annual questionnaires. Blood pressure and A1C level were measured annually in the EDIC study. Plasma lipid concentration (cholesterol and triglycerides) was measured biannually during the EDIC study.

### AGEs in Skin Biopsies Samples

The AGE measurements used for this prospective longitudinal study have been previously described in detail (7). Briefly recapitulated, the skin biopsy sample used in these studies was stored since 1993 at −80°C under argon. Insoluble collagen was prepared and enzymatically digested to release the free glycation products that were quantitated by liquid chromatography mass spectrometry using isotope dilution assays for GSPNE, CEL, G-H1, and MG-H1. This set of AGEs is referred to as the “added set” (*n* = 4) in contrast to the “original set” of six AGEs/markers that included collagen fluorescence, acid solubility, pepsin digestibility, CML, pentosidine, and FUR (5,6).

Although native FL was automatically included in the new analytical system, we report here only the values of the originally measured acid hydrolytic conversion product FUR, but also provide some details on how FUR versus

**Table 2—Univariate analysis of risk factors versus further worsening retinopathy, new onset of neuropathy, or new onset of neuropathy during EDIC study#**

Characteristics	Three-step or more ETDRS scale progression from DCCT closeout through EDIC study years 13–16		Development of microalbuminuria or worse (AER ≥ 40 mg/24 h) from DCCT closeout through EDIC study years 15–16		Development of confirmed clinical neuropathy or abnormal autonomic function from DCCT closeout to EDIC study years 13–14 or 16–17*				
	Event (N = 91)	Nonevent (N = 122)	P value	Event (N = 50)	Nonevent (N = 145)	P value	Event (N = 74)	Nonevent (N = 91)	P value
<b>Demographic</b>									
Age at EDIC study baseline (years)	33.6 ± 7.0	34.3 ± 6.6	0.52	32.6 ± 6.9	34.5 ± 6.6	0.09	34.7 ± 6.3	32.5 ± 6.5	0.04
Women (%)	52.8	43.3	0.18	58.0	44.8	0.11	44.6	48.4	0.63
Diabetes duration at EDIC study baseline (years)	10.5 ± 4.4	11.0 ± 5.0	0.64	10.1 ± 3.9	10.7 ± 4.8	0.70	10.8 ± 4.6	10.4 ± 4.7	0.52
DCCT treatment group intensives (%)	44.0	65.5	0.002	48.0	60.7	0.12	56.8	56.0	0.93
<b>Glycemia control</b>									
DCCT mean A1C %	8.7 ± 1.7	7.5 ± 1.3	<0.0001	8.8 ± 1.6	7.7 ± 1.4	<0.0001	8.3 ± 1.7	7.7 ± 1.3	0.04
mmol/mol	72 ± 18.6	58 ± 14.2		73 ± 17.5	61 ± 15.3		67 ± 18.6	61 ± 14.2	
EDIC study mean A1C up to EDIC study year 16 %	8.6 ± 1.2	7.6 ± 0.9	<0.0001	8.9 ± 1.2	7.7 ± 1.0	<0.0001	8.3 ± 1.3	7.7 ± 0.9	0.004
mmol/mol	70 ± 13.1	60 ± 9.8		74 ± 13.1	61 ± 10.9		67 ± 14.2	61 ± 9.8	
<b>Medical at EDIC study baseline</b>									
Mean blood pressure (mmHg)	90.2 ± 10	88.5 ± 7.9	0.23	90.9 ± 8.7	88.4 ± 8.2	0.10	89.9 ± 7.4	87.9 ± 8.7	0.19
Hypertension (%)†	8.8	1.6	0.017	8.0	3.5	0.19	2.7	5.5	0.38
Triglycerides (mg/dL)	81.3 ± 40	77.6 ± 44	0.39	82.9 ± 42	74.5 ± 40	0.21	86.3 ± 57	75.8 ± 34	0.63
HDL cholesterol (mg/dL)	52.9 ± 13	50.3 ± 13	0.15	52.1 ± 12	51.5 ± 13	0.74	50.8 ± 15	50.6 ± 11	0.67
LDL cholesterol (mg/dL)	113 ± 28	108 ± 28	0.16	114 ± 27	108 ± 28	0.08	119 ± 31	106 ± 27	0.004
Overweight (%)‡	55.0	54.1	0.90	62.0	53.1	0.28	60.8	49.5	0.15
Smoker (%)	28.6	18.9	0.10	26.0	18.6	0.27	29.7	18.7	0.10
<b>Retinopathy at EDIC study baseline (%)</b>									
No retinopathy (10/10)	30.8	27.9		20.0	34.5		21.6	35.2	
Microaneurysm only (20/<20)	30.8	41.0	0.22	34.0	39.3	0.008	40.5	42.9	0.01
Mild NPDR (35/<35)	20.0	24.6		30.0	17.9		24.3	15.4	
Moderate NPDR or worse (43/≤43+)	18.9	6.6		16.0	8.3		13.5	6.6	
<b>Renal at EDIC study baseline</b>									
AER (mg/24 h)	10.1	7.2	0.0002	10.1	7.2	0.0009	101.1	7.2	0.007
Median (interquartiles)	(6, 20)	(4, 12)		(7, 17)	(4, 10)		(6, 20)	(6, 12)	
AER ≥ 40 (%)	14.3	4.1	0.008	0 ~	0 ~	n/a	16.2	2.2	0.001
<b>Neuropathy at EDIC study baseline (%)</b>									
Confirmed clinical neuropathy	12.2	6.6	0.15	12.0	7.6	0.35	0	0	n/a
Abnormal autonomic function	15.1	2.8	0.003	9.1	5.7	0.44	0	0	n/a
<b>Skin collagens at EDIC study baseline</b>									
FUR (pmol/mg)	878 ± 251	686 ± 179	<0.0001	875 ± 236	706 ± 194	<0.0001	821 ± 233	691 ± 212	0.0001
GML (pmol/mg)	580 ± 138	503 ± 117	<0.0001	569 ± 125	513 ± 130	0.009	571 ± 119	502 ± 130	<0.0001
Pentosidine (pmol/mg)	27.7 ± 7.6	24.6 ± 6.9	0.002	27.5 ± 8.0	24.8 ± 6.8	0.02	27.1 ± 6.9	24.0 ± 6.7	0.005

Continued on p. 270



with greater progression of retinopathy ( $P < 0.0001$ ), nephropathy ( $<0.0001$ ), and neuropathy ( $P = 0.04-0.004$ ). Of note, the EDIC study baseline AER was significantly greater in those participants who later showed progression of all three complications during the EDIC study (each  $P < 0.008$ ), whereas the degree of retinopathy at EDIC study baseline was associated only with the progression of nephropathy and neuropathy (each  $P \leq 0.01$ ).

All AGEs except for CEL and G-H1 were associated with progression of retinopathy (each  $P \leq 0.01$ ) (Fig. 1); all except acid-soluble collagen, CEL, G-H1, and MG-H1 were associated with progression of nephropathy (Fig. 2) (each  $P \leq 0.02$ ); and all except acid-soluble collagen, CEL, and G-H1 were significantly associated with progression of neuropathy (Fig. 3) (each  $P \leq 0.02$ ). All of these remained significant after the Benjamini and Hochberg FDR adjustment (each FDR adjusted  $P < 0.05$ ) (20). As previously reported, FUR, CML, pentosidine, relative fluorescence, and pepsin solubility (negatively) were significantly

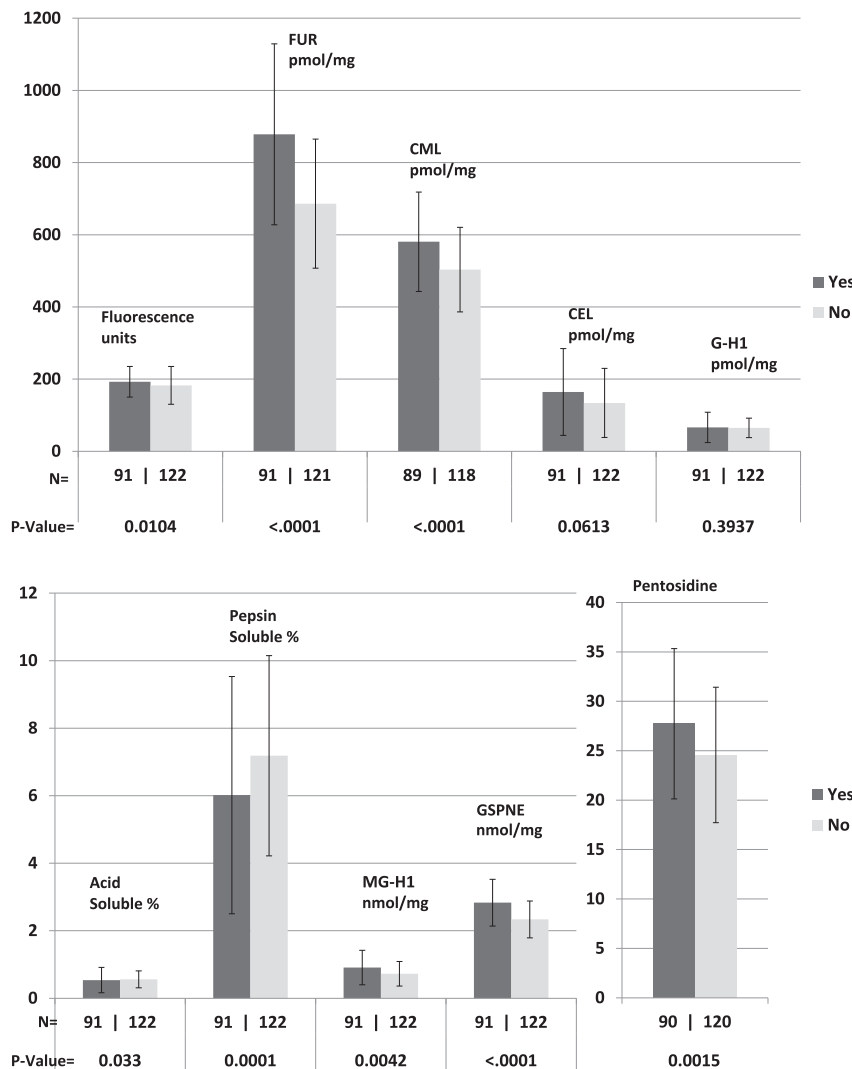
associated with all three complications, but only GSPNE of the four newly measured AGEs was uniformly associated with future progression of all complications ( $P < 0.0001$ ).

**Multivariable Logistic Regression Analyses**

Tables 3–5 present multivariable logistic regression analyses of covariates associated, respectively, with progression of retinopathy, nephropathy, and neuropathy.

**Retinopathy**

The complete panel comprising the original set and the new set of AGEs was significantly associated with progression of retinopathy 16 years after DCCT closeout and remained so after adjustment for mean DCCT A1C level, mean EDIC study A1C level, and all the other significant factors from univariate analyses listed in Tables 2 and 3 (treatment group, AER, and autonomic neuropathy). The same was true for the panel of six original AGEs, and backward-selected AGEs FUR and GSPNE, jointly or



**Figure 1**—AGE levels in those with (yes) or without (no) three-step progression of retinopathy on the ETDRS scale from DCCT closeout up to EDIC study year 16.



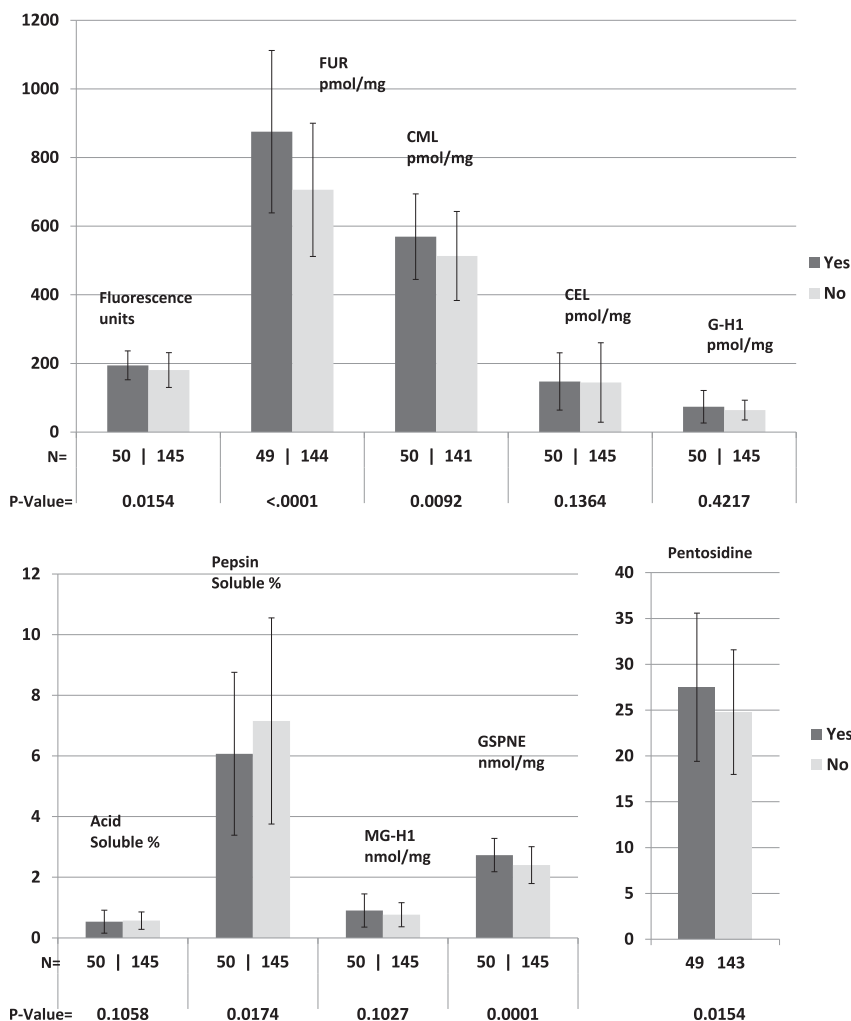


Figure 2—AGE levels in those with (yes) or without (no) development of AER >40 mg/24 h from DCCT closeout to EDIC study years 15–16.

individually. The four added AGEs strengthened the association between the original AGEs and retinopathy (LRT  $P = 0.02$ ). Interestingly, the mean DCCT A1C effect on retinopathy progression during the EDIC study lost significance when adjusted for the whole panel of AGEs, for FUR alone, or for the combination of FUR and GSPNE (i.e., glycated collagen and its downstream cross-linked product).

**Nephropathy**

The whole panel of 10 AGEs and the panel of 6 original AGEs were both associated with the progression of nephropathy 15–16 years after DCCT closeout (Table 4). However, the added AGEs did not strengthen this association ( $P = 0.59$ ). FUR was selected as the strongest predictor of nephropathy, which remained significantly associated after adjustment for mean DCCT and mean EDIC study A1C levels, whereas the mean DCCT A1C effect lost significance when adjusted for FUR or the whole panel of AGEs. The nephropathy analysis was repeated by using the outcome of a sustained AER of  $\geq 30$  mg/24 h (Supplementary Tables 1 and 2). The results were almost identical to what was reported for AER

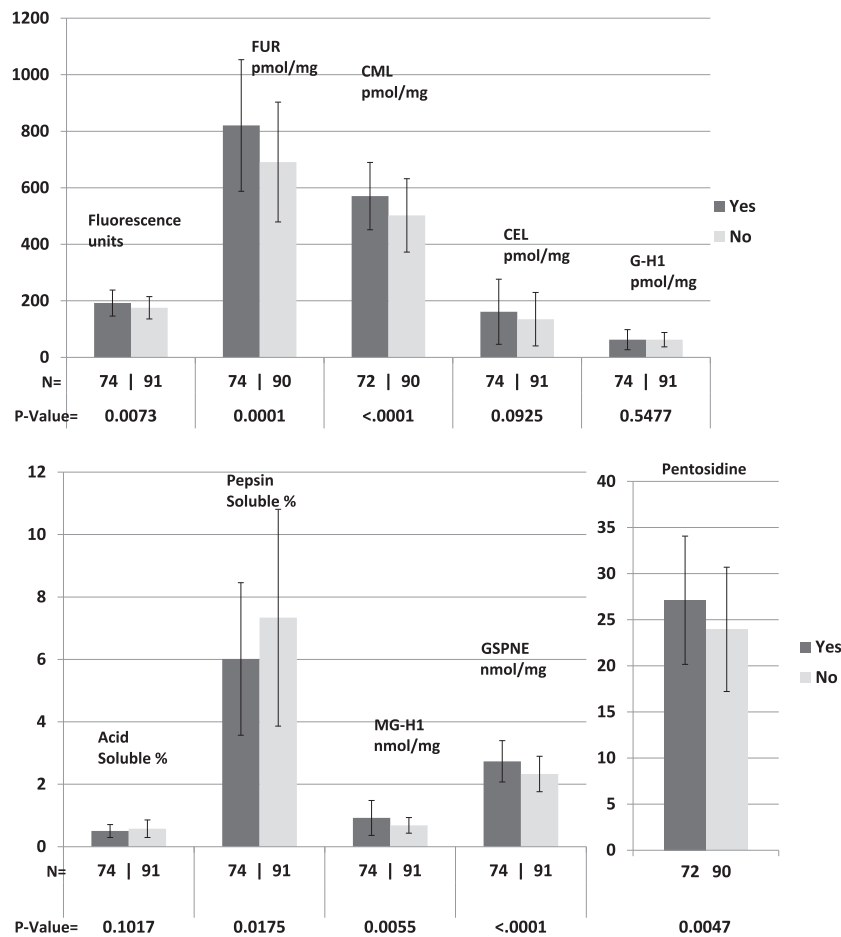
$\geq 40$  mg/24 h, which confirmed the robustness of these findings.

**Neuropathy**

The entire panel of 10 AGEs, the panel of 6 original AGEs, the combination of backward-selected AGEs FUR and MG-H1, and both of these AGEs individually, remained associated with the progression of neuropathy in the EDIC study after adjustment for mean DCCT A1C and mean EDIC study A1C levels (Table 5). The four new AGEs strengthened the association between the six original AGEs and neuropathy (LRT  $P = 0.04$ ). Moreover, the effect of mean DCCT A1C level on neuropathy progression lost significance when adjusted for FUR alone or in combination with MG-H1 and when adjusted for the whole panel of AGEs.

**FUR Versus FL and Associations Among AGEs and With A1C**

We have reported in this study the 13–15 year EDIC study data on FUR from the original set instead of FL, its native precursor that was automatically included in the



**Figure 3**—AGE levels in those with (yes) or without (no) development of confirmed clinical neuropathy or abnormal autonomic nervous system function from DCCT closeout to EDIC study years 13–14.

analytical procedure. Both markers performed about the same in the Spearman correlation analysis with other AGEs (Supplementary Table 3). As expected, the two markers correlated highly with each other ( $R^2 = 0.70$ ,  $P < 0.001$ ). The  $R^2$  values with other AGEs were very similar, in general slightly favoring FUR over FL, except for the correlation with GSPNE (0.52 vs. 0.56) and MG-H1 (0.13 vs. 0.26), whereby only FL was significant ( $P < 0.01$ ). As expected, FUR correlated better with past (DCCT) than prospective (EDIC study) mean glycemia levels, as reflected by A1C values (0.72 vs. 0.29, both  $P < 0.001$ ). Of interest is that collagen pepsin solubility was highly inversely correlated with most other markers, in particular with the matrix cross-link GSPNE, which is the most abundant AGE in skin. GSPNE was the glycation product most strongly correlated with the other AGEs, followed by FUR and MG-H1. FUR ( $r = 0.72$ ,  $P < 0.0001$ ) was more strongly associated with mean DCCT A1C level than FL ( $r = 0.48$ ,  $P < 0.0001$ ). CEL, G-H1, and MG-H1 were not correlated with mean DCCT A1C level. CML correlated with each component of the original set of AGEs, as previously reported, and also correlated with GSPNE ( $r = 0.50$ ,  $P < 0.0001$ ) and with DCCT mean A1C

level ( $r = 0.38$ ,  $P < 0.0001$ ) but did not correlate with EDIC study mean A1C level.

As for correlation with complications, our previous study on association with the progression of complications during the DCCT up to the time of the biopsy (7) revealed that the order of individual strength of association (entropy  $R^2$ ) for sustained three-step progression was FL > FUR (each  $P < 0.05$ ), but for sustained progression greater than or equal to 3 microaneurysms, it was FUR > FL ( $P < 0.05$ ); for nephropathy, it was FUR > FL (each  $P < 0.05$ ); and for neuropathy, it was FUR >>> FL (each  $P < 0.05$ ). For the future progression of complications during the EDIC study, we found only trivial differences in the  $\chi^2$  and  $R^2$  effects, leading to the conclusion that both forms of the Amadori product behave very similarly.

## DISCUSSION

In univariate analysis of eight AGEs and two collagen solubility markers, four of which we have measured for the first time in our original DCCT biopsy samples (i.e., GSPNE, CEL, G-H1, and MG-H1), GSPNE stands out in its significant association with three-step ETDRS



**Table 3—Summary of multivariable logistic regressions of AGE associations with the prevalence of retinopathy without and with adjustment for A1C, and vice versa**

Covariate effects evaluated*	df	$\chi^2$	P value	Entropy $R^2$	OR (95% CI)†
<b>All AGEs (FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE)</b>					
Unadjusted	10	53.7	<0.0001	0.19	
Adjusted for					
DCCT mean A1C	10	27.3	0.002	0.10	
EDIC study mean A1C	10	46.2	<0.0001	0.16	
<b>Original AGEs published in 2005 (FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen)</b>					
Unadjusted	6	42.4	<0.0001	0.15	
Adjusted for					
DCCT mean A1C	6	16.3	0.01	0.06	
EDIC study mean A1C	6	33.9	<0.0001	0.12	
<b>Backward selected all AGEs (FUR, GSPNE)</b>					
Unadjusted	2	45.5	<0.0001	0.16	
Adjusted for					
DCCT mean A1C	2	17.1	0.0002	0.06	
EDIC study mean A1C	2	32.9	<0.0001	0.12	
CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1	2	30.5	<0.0001	0.11	
Selected factors‡	2	14.5	0.0006	0.06	
<b>FUR</b>					
Unadjusted	1	37.4	<0.0001	0.13	2.6 (1.8–3.6)
Adjusted for					
DCCT mean A1C	1	9.8	0.002	0.03	2.0 (1.3–3.2)
EDIC study mean A1C	1	25.1	<0.0001	0.09	2.5 (1.6–3.2)
Selected factors‡	1	5.9	0.01	0.03	2.5 (1.3–4.0)
<b>GSPNE</b>					
Unadjusted	1	31.8	<0.0001	0.11	2.4 (1.7–3.4)
Adjusted for					
DCCT mean A1C	1	12.2	0.0005	0.04	2.0 (1.4–2.9)
EDIC study mean A1C	1	24.2	<0.0001	0.08	2.3 (1.6–3.2)
Selected factors‡	1	11.5	0.0007	0.05	2.2 (1.3–3.5)
<b>DCCT mean A1C effect</b>					
Unadjusted	1	30.4	<0.0001	0.10	2.3 (1.7–3.3)
Adjusted for					
FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE	1	0.23	0.64	0.0	1.1 (0.7–1.9)
Backward-selected AGEs (FUR and GSPNE)	1	0.86	0.35	0.0	1.3 (0.8–2.0)
FUR alone	1	1.65	0.20	0.0	1.4 (0.9–2.2)
GSPNE alone	1	10.8	0.001	0.04	1.8 (1.3–2.6)
<b>EDIC study mean A1C effect</b>					
Unadjusted	1	34.7	<0.0001	0.12	2.4 (1.7–3.3)
Adjusted for					
FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE	1	24.3	<0.0001	0.09	2.4 (1.6–3.6)
Backward-selected AGEs (FUR and GSPNE)	1	20.0	<0.0001	0.07	2.1 (1.5–3.0)

\*Within each block, multiple model effects are presented using the set of variables named within each block. For the first block, the joint effect of all 10 AGEs is assessed. The unadjusted model shows the effect of the named variables without adjustment for other factors (e.g., the effect of the 10 AGEs in combination). This is followed by the effect of the covariates after adjustment for one or more specified factors, each from a separate model (e.g., the effect of the 10 AGEs in a model adjusted for the DCCT mean A1C and in a model adjusted for the EDIC study mean A1C). †Odds ratio (OR) is based on 1 SD increase in the continuous variables (DCCT mean A1C 1.6% [17.5 mmol/mol], EDIC study mean A1C 1.1% [12.0 mmol/mol], FUR 231, GSPNE 0.66, MG-H1 0.44). ‡A model adjusted for the following factors that were nominally, statistically, and significantly associated with the outcome in univariate analyses in Table 2: DCCT mean A1C, EDIC study mean A1C up to year 16, DCCT treatment group, and both the log(AER) and abnormal autonomic function at EDIC study baseline.

worsening of retinopathy, the development of microalbuminuria, and the development of neuropathy from the closeout of DCCT to 13–16 years later in the EDIC

study. In multivariable analysis using backward selection, GSPNE was associated with the subsequent development of retinopathy even after adjustment for DCCT mean A1C

**Table 4—Summary of multivariable logistic regressions of AGE associations with the prevalence of nephropathy without and with adjustment for A1C level, and vice versa**

Covariate effects evaluated*	df	$\chi^2$	P value	Entropy $R^2$	OR (95% CI)†
<b>All AGEs (FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE)</b>					
Unadjusted	10	24.0	0.008	0.11	
Adjusted for					
DCCT mean A1C	10	9.6	0.48	0.04	
EDIC study mean A1C	10	18.1	0.05	0.08	
<b>Original AGEs published in 2005 (FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen)</b>					
Unadjusted	6	21.2	0.002	0.10	
Adjusted for					
DCCT mean A1C	6	6.8	0.34	0.03	
EDIC study mean A1C	6	15.3	0.02	0.07	
<b>Backward-selected AGEs (FUR)</b>					
Unadjusted	1	21.5	<0.0001	0.10	2.3 (1.6–3.3)
Adjusted for					
DCCT mean A1C	1	5.1	0.02	0.02	2.0 (1.0–3.2)
EDIC study mean A1C	1	13.1	0.0003	0.06	2.0 (1.3–2.5)
CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE	1	11.7	0.0006	0.05	2.0 (1.0–3.2)
Selected factors‡	1	3.9	0.049	0.02	2.5 (1.3–5.0)
<b>DCCT mean A1C effect</b>					
Unadjusted	1	18.8	<0.0001	0.08	3.0 (1.5–2.1)
Adjusted for					
FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE	1	0.8	0.38	0.0	1.3 (0.7–2.2)
Backward-selected AGEs (FUR)	1	1.4	0.23	0.01	1.4 (0.8–2.3)
<b>EDIC study mean A1C effect</b>					
Unadjusted	1	44.0	<0.0001	0.20	3.4 (2.2–5.1)
Adjusted for					
FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE	1	34.1	<0.0001	0.16	3.2 (2.0–5.0)
Backward-selected AGEs (FUR)	1	33.8	<0.0001	0.15	3.1 (2.0–4.8)

\*Within each block, multiple model effects are presented using the set of variables named within each block. For the first block, the joint effect of all 10 AGEs is assessed. The unadjusted model shows the effect of the named variables without adjustment for other factors (e.g., the effect of the 10 AGEs in combination). This is followed by the effect of the covariates after adjustment for one or more specified factors, each from a separate model (e.g., the effect of the 10 AGEs in a model adjusted for the DCCT mean A1C and in a model adjusted for the EDIC study mean A1C). †Odds ratio (OR) is based on 1 SD increase in the continuous variables (DCCT mean A1C 1.6% [17.5 mmol/mol], EDIC study mean A1C 1.1% [12.0 mmol/mol], FUR 231, GSPNE 0.66, MG-H1 0.44). ‡A model adjusted for the following factors that were nominally, statistically, and significantly associated with the outcome in univariate analyses in Table 2: DCCT mean A1C, EDIC study mean A1C up to year 16, and both retinopathy and log(AER) at EDIC study baseline.

and EDIC study mean A1C levels, as well as DCCT treatment group and log AER at EDIC study baseline (Table 3). In addition, it is noteworthy that the content of GSPNE in skin collagen from people with type 1 diabetes is 3–4 times higher than that of FUR, CML, or MG-H1; 17 times higher than that of CEL; 42 times higher than that of G-H1; and 100 times higher than that of pentosidine. GSPNE is also quantitatively both the single most prevalent AGE and protein cross-link, and it is exclusively derived from glucose. These facts support GSPNE as perhaps being the one AGE that plays the most important role in the pathogenesis of retinopathy from hyperglycemia. This is not to deny a role for other AGEs either individually or in combination.

In univariate analysis, FUR, CML, and pentosidine were associated with all three progressive complications, as previously reported (6), and FUR remained

significant after adjustment for DCCT mean A1C and for EDIC study mean A1C levels. MG-H1, derived from methylglyoxal, was also strongly associated with retinopathy ( $P = 0.004$ ) and neuropathy ( $P = 0.006$ ), but was not significantly associated with nephropathy ( $P = 0.1$ ).

Not surprisingly, there were numerous correlations between many pairs of AGEs, and between AGEs and DCCT mean A1C level (Supplementary Table 3), which expressed the glycemic exposure prior to the measurement of the AGEs. Again, the correlations between GSPNE and each of the other AGEs as well as with DCCT mean A1C level were most often the strongest of any single AGE. FUR, a measure of glycated collagen, correlated most strongly with DCCT mean A1C level ( $r = 0.72$ ), while the AGE MG-H1, formed from methylglyoxal originating mainly from the degradation of cellular

**Table 5—Summary of multivariable logistic regressions of AGE associations with the prevalence of neuropathy without and with adjustment for A1C, and vice versa**

Covariate effects evaluated*	df	$\chi^2$	P value	Entropy $R^2$	OR (95% CI)†
<b>All AGEs (FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE)</b>					
Unadjusted	10	29.7	0.0009	0.13	
Adjusted for					
DCCT mean A1C	10	25.4	0.005	0.11	
EDIC study mean A1C	10	28.3	0.002	0.13	
<b>Original AGEs published in 2005 (FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen)</b>					
Unadjusted	6	19.6	0.003	0.09	
Adjusted for					
DCCT mean A1C	6	15.2	0.02	0.07	
EDIC study mean A1C	6	16.4	0.01	0.07	
<b>Backward-selected AGEs (MG-H1, FUR)</b>					
Unadjusted	2	26.5	<0.0001	0.12	
Adjusted for					
DCCT mean A1C	2	21.1	<0.0001	0.09	
EDIC study mean A1C	2	24.0	<0.0001	0.11	
CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1	2	8.2	0.02	0.04	
Selected factors‡	2	18.5	<0.0001	0.08	
<b>MG-H1</b>					
Unadjusted	1	13.9	0.0002	0.06	2.0 (1.3–3.1)
Adjusted for					
DCCT mean A1C	1	13.7	0.0002	0.06	2.0 (1.3–3.0)
EDIC study mean A1C	1	18.3	<0.0001	0.08	2.3 (1.5–3.5)
Selected factors‡	1	16.1	<0.0001	0.07	2.3 (1.4–3.7)
<b>FUR</b>					
Unadjusted	1	13.3	0.0003	0.06	1.8 (1.3–2.6)
Adjusted for					
DCCT mean A1C	1	7.8	0.005	0.03	2.0 (1.3–3.2)
EDIC study mean A1C	1	7.8	0.005	0.03	1.6 (1.3–2.5)
Selected factors‡	1	2.3	0.13	0.01	1.6 (0.8–3.2)
<b>DCCT mean A1C effect</b>					
Unadjusted	1	5.7	0.02	0.03	1.5 (1.1–2.1)
Adjusted for					
FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE	1	1.0	0.31	0.0	0.7 (0.4–1.3)
Backward-selected AGEs (FUR, MG-H1)	1	0.4	0.51	0.0	0.8 (0.5–1.5)
FUR	1	0.3	0.56	0.0	0.9 (0.5–1.5)
MG-H1	1	5.5	0.02	0.02	1.5 (1.1–2.1)
<b>EDIC study mean A1C effect</b>					
Unadjusted	1	10.0	0.002	0.04	1.7 (1.2–2.3)
Adjusted for					
FUR, CML, pentosidine, fluorescence, acid-soluble collagen, pepsin-soluble collagen, CEL, G-H1, MG-H1, GSPNE	1	7.4	0.007	0.03	1.6 (1.1–2.4)
Backward-selected AGEs (FUR, MG-H1)	1	7.7	0.006	0.03	1.7 (1.1–2.5)

\*Within each block, multiple model effects are presented using the set of variables named within each block. For the first block, the joint effect of all 10 AGEs is assessed. The unadjusted model shows the effect of the named variables without adjustment for other factors (e.g., the effect of the 10 AGEs in combination). This is followed by the effect of the covariates after adjustment for one or more specified factors, each from a separate model (e.g., the effect of the 10 AGEs in a model adjusted for the DCCT mean A1C and in a model adjusted for the EDIC study mean A1C). †Odds ratio (OR) is based on 1 SD increase in the continuous variables (DCCT mean A1C 1.6% [17.5 mmol/mol], EDIC study mean A1C 1.1% [12.0 mmol/mol], FUR 231, GSPNE 0.66, MG-H1 0.44). ‡A model adjusted for the following factors that were nominally, statistically, and significantly associated with the outcome in univariate analyses in Table 2: DCCT mean A1C, EDIC study mean A1C up to year 16, and also age, LDL, and log(AER) at EDIC study baseline.

triosephosphate and influenced by glycolytic activity (21,22), did not correlate at all with mean DCCT A1C level. Within the panel of 10 AGEs reported here, 2 were indirect indices of cross-linking (i.e., acid solubility and pepsin solubility),

with each representing physicochemical properties that reflect the incorporation of AGEs into tissue proteins.

Although GSPNE has been known since 1999 (23), its precise role in the development of diabetes complications

remains to be understood. Incorporation of GSPNE may decrease the turnover of collagen and increase its accumulation, perhaps contributing to the thickening of basement membranes (24) in the retina, kidney, and peripheral nerves in addition to hyperglycemia-mediated increased extracellular matrix deposition (25). Second, the necessity of arginine residues for the formation of GSPNE (Fig. 1) may compete with integrin-binding sites to basement membrane (23,26), and the resultant loss of proper attachment of endothelial cells to basement membrane may promote programmed death of these cells (27).

An unexpected finding was that the methylglyoxal AGE MG-H1 remained strongly associated with neuropathy progression despite adjustment for all other risk factors. This provides crucial support for the recently reported association between neuropathy and methylglyoxal in 27 participants of the Oslo Study (28). These clinical findings, together with preclinical data, provide a rationale for in-depth studies on the role of methylglyoxal in diabetic neuropathy.

The strengths of this study are the availability of tissue specimens from an exceptionally well-characterized type 1 diabetes cohort with repeated measurements of retinopathy, nephropathy, and neuropathy by the latest and most modern validated methods for up to 17 years after the skin biopsy samples were obtained and stored. There also are limitations to the study. The data are fundamentally associative; even though the AGE measurements preceded the subsequent development of complications and have a predictive quality, this by itself does not prove a causal role. It is conceivable that an antecedent pathogenic factor induces independently both the complications and AGEs in parallel fashion. It is also not certain that qualitative or quantitative findings in skin collagen AGEs reflect those of retinal, renal, or neural tissue.

In conclusion, skin collagen AGEs are strongly associated with the future development of diabetic retinopathy, nephropathy, and neuropathy up to 17 years later. GSPNE is the single most prevalent measured AGE in human skin collagen and is systematically most associated with the progression of these complications, along with its immediate glucose-derived precursor, FL, judged herein by the presence of the surrogate marker FUR.

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