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ORIGINAL ARTICLE

Detection of diabetic nephropathy from advanced glycation endproducts (AGEs) differs in plasma and urine, and is dependent on the method of preparation

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Abstract Increased advanced glycation endproducts (AGEs) and oxidation products (OPs) have been proposed as pathogenic for diabetic nephropathy (DN). We investigated the relationship between AGEs and OPs measured in different plasma and urine preparations, and progression of DN in 103 young, normoalbuminuric, normotensive participants with type 1 diabetes in the Natural History of Diabetic Nephropathy Study. The primary endpoint was electron microscopy-measured change in glomerular basement membrane (GBM) width from baseline to 5 years; change in mesangial fractional volume was a secondary endpoint. Fast progressors (FP) were defined as the upper quartile ($n = 24$) of rate of GBM thickening; slow progressors (SP) were the remainder ($n = 79$). Four AGEs [3-deoxyglucosone and methylglyoxal hydroimidazolones (DG3H1, MGH1) and carboxymethyl and ethyl lysine (CML, CEL)], and two oxidation products methionine sulfoxide and aminoadipic acid were measured by liquid chromatography, triple quadrupole mass spectrometry. Measurements were done on 10 K plasma filtrates and plasma proteolytic digests (PPD) at year 5, and at four time points over 5 years for urinary 10 K filtrates. Urinary filtrate CEL levels were significantly higher in FP, but not after adjustment for HbA1c, sex, and duration of diabetes. MGHI, CEL, and CML plasma filtrate levels were significantly higher in FP relative to SP ($p < 0.05$). In PPD, only MGHI showed borderline significantly higher levels in FP relative to SP ($p = 0.067$), while no other product showed correlation. AGE and OP measurements were not correlated with mesangial expansion. In plasma filtrates, HbA1c at year 5 accounted for 4.7 % of the variation in GBM width. The proportion of variation in GBM width was increased to 11.6 % when MGHI, CEL, and CML were added to the model (6.9 % increase).

Keywords Diabetic nephropathy · Diabetic complications · Advanced glycation endproducts · Oxidation end-products · Glycation · Oxidation · Biomarkers · Mass spectrometry

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Introduction

Although several large-scale studies have clearly demonstrated that intensive glucose control plays a role in preventing or delaying diabetic complications (DCCT Research Group 1993; Turner et al. 1999), effective new biomarkers for early detection of clinically silent kidney damage have not been established (Mogensen and Christensen 1984; Caramori et al. 2000; Drummond et al. 2002; Perkins and Krolewski 2009). Lack of progress in this field is due to multiple factors, including sub-optimal measurement

methods or lack of specificity of biomarkers, incomplete knowledge of types of samples to use or how to process them, or inadequate characterization of outcome phenotype in the populations studied (Monnier et al. 2005; Beisswenger 2012). Our inability to detect the risk of complications leads to a situation where many individuals with diabetes already have significant disease at the time of diagnosis after many years of silent evolution (Cefalu 2008; Dluhy and McMahon 2008). As a consequence, intensive therapies are less likely to be instituted during the early stages when complications are potentially preventable or reversible. Consequently we frequently delay therapies until frank clinically observable signs or symptoms occur.

Our current major biomarker for progression of diabetic nephropathy (DN), the appearance of albuminuria on regular examinations, is often unable to identify those at greatest risk during the long 10–20 years "silent phase" when evolving structural damage is not otherwise clinically apparent (Fioretto et al. 1999). By the time these markers become positive, substantial glomerular damage can be present. In addition, angiotensin converting enzyme inhibitor (ACEI) and angiotensin receptor blockers (ARBs), instituted before albuminuria is detected, are unable to slow progression of structural glomerular lesions (Mauer et al. 2009).

HbA1c as a tool to predict diabetic complications also has significant shortcomings, including its relative insensitivity to glycemic fluctuations (Beisswenger et al. 2001; Rohlfing et al. 2002; Ceriello et al. 2004; Monnier and Colette 2006), and the inability of this early chemical reaction product (Amadori product) to detect variable production of more complex glycation/oxidation products that may induce vascular damage. Based on the limitations of the currently used biomarkers, our clinical treatment decisions have to be made on the premise that all diabetic patients are equally susceptible to complications when we attempt to initiate early aggressive goals for treatment of glycemia. New biomarkers of risk for progression to DN are needed that improve prediction independent of microalbuminuria and HbA1c.

The task of predicting DN is further complicated by the variable propensity to diabetic complications among individuals with diabetes (Seaquist et al. 1989; DCCT Research Group 1997; Fioretto et al. 1999). While poor glycemic control is clearly a DN risk factor (DCCT/EDIC Complications Research Group 2002), accelerated DN may occur in some individuals with modest hyperglycemia, while others never progress in spite of poor glycemic control over many years (Andersen et al. 1983; Krolewski et al. 1987). Family studies show clustering of the risk of DN (Prager et al. 1981; Seaquist et al. 1989; Pettitt et al. 1990; DCCT Research Group 1997) and there is concordance for the severity and patterns of diabetic glomerular structural lesions among type 1 diabetic sibling pairs (Fioretto et al. 1999). Further support for a variable response of the kidney to hyperglycemia comes from work showing that other less well-defined variables, of equal or greater importance to glycemia, strongly influence nephropathy risk (Caramori et al. 2002).

New insights into individual propensity to DN is provided by the observations that increased cellular production of the highly reactive alpha dicarbonyl, methylglyoxal, and increased oxidative stress occur in patients with rapid DN progression (Beisswenger et al. 2005, 2008). Since these dicarbonyl and oxidative pathways ultimately result in specific advanced glycation and oxidative end-products (Ahmed et al. 2005a, b), we have investigated these products in serum, plasma, and urine samples, processed in different ways, as DN predictors in patients with type 1 diabetes (T1DM). (Drexel et al. 1987; Drummond et al. 2002, 2003).

Methods

Study population

We utilized blood and urine samples from 103 normoalbuminuric subjects participating in the Natural History of Diabetic Nephropathy Study (NHS). This study was designed to examine the early development of DN in subjects with T1DM, as determined by electron-microscopic quantification of two research renal biopsies performed 5 years apart (Drummond et al. 2002, 2003). Analyses of the baseline data cohort from NHS showed that biopsy changes consistent with early DN lesions could be present within a few years of T1DM onset, well before clinical evidence of their presence is discernable, and that these baseline structural changes were predictive of the later development of persistent microalbuminuria (MA). (M. Mauer, personal communication). The characteristics of this young, normoalbuminuric T1DM population is shown in Table 1.

Table 1 Characteristics of NHS population at baseline

	Mean ± SD
Number of subjects	103
Age	17.6 ± 7.4 years
Diabetes duration	8.3 ± 4.9 years
Mean albumin excretion rate (AER)	6.5 ± 8.3 μg/min
Mean baseline HbA1c	8.6 ± 1.3 % (70 ± 10.6 mmol/mol)[a]
Glomerular filtration rate	146 ± 23 mL/min/1.73 m^2
Systolic blood pressure	115 ± 11 mmHg
Diastolic blood pressure	63 ± 9 mmHg

[a] Mean A1c from baseline to 5 years did not change significantly

Our primary renal endpoint in this population was defined as change in glomerular basement membrane (GBM) width from baseline to 5 years, measured in electron micrographs of renal biopsies. Mesangial fractional volume was also measured as a secondary endpoint. Fast progressors (FP) were defined as the upper quartile ($n = 24$) of GBM thickening and slow progressors (SP) as the remainder ($n = 79$).

Sample processing

Serum and urine samples were collected from 1997 to 2000 and processed at multiple sites. Serum was allowed to clot at room temperature for undefined duration and temperature conditions. They were then stored for variable times at −20 °C and later at −80 °C until analysis.

Glycation and Oxidative end-products specified below were measured on serum, plasma, and urine samples collected from NHS subjects.

Serum and urine samples were collected at 4–6 time points during the 5 year study. Plasma samples were collected at the 5-year NHS visit by a rigorous protocol as follows: Blood was collected in EDTA containing tubes and immediately iced and centrifuged. Following centrifugation, plasma was immediately separated from RBCs, snap-frozen on dry ice, and stored at −80 °C until analyses were performed.

We performed liquid chromatography/triple quadrupole mass spectrometry (LC–MS/MS) analyses on the plasma and serum "free adduct", prepared as the filtrate following centrifugation through 10 K cut-off Amicon™ filters. 10 K filtrates were also prepared on urine samples collected at four spaced time points over 5 years. Adduct residues chemically bound to plasma or serum proteins, some of which are acid labile, were also determined after exhaustive sequential enzymatic digests with pepsin, Pronase E, and aminopeptidase/prolidase (50 µg protein equivalent) under nitrogen as previously described (Ahmed et al. 2005a), with controls for protease autolysis.

Biomarkers measured

Nine glycation and oxidation end products were measured by LC–MS/MS, utilizing internal stable heavy isotope substituted standards. An Agilent Model 6410 Triple Quadrupole MS System with 1200 Rapid Resolution LC System was used and we have developed methods for the concurrent quantitative measurement of biomarkers indicative of protein glycation, oxidation, and nitrosative damage by employing a single 2.0 × 250 mm Synergy 4 micron 80A column (Phenomenex, USA) with a mobile phase of methanol/H_2O gradient with 0.29 % heptafluorobutyric acid (HFBA), and a total analysis time of 60 min.

Advanced glycation endproducts (AGEs) measured included carboxymethyl lysine (CML), carboxyethyl lysine (CEL), glyoxal hydroimidazolone (G-HI), methylglyoxal hydroimidazolone (MG-HI), and 3-deoxyglucosone hydroimidazolone (3DG-H). CEL and MGHI are methylglyoxal derived AGEs of lysine and arginine, respectively. Oxidative endproducts (OP) included methionine sulfoxide (MethSO), 3-Nitrotyrosine (3-NT), 2-aminoadipic acid (AAA), and dityrosine.

Standards

MethSO and DiTyr were synthesized, purified, and validated in our laboratory. MethSO and its heavy deuterium standard were synthesized and purified using the procedure by Roper and McIlwain (1948). Structural confirmation, purity of >95 %, and concentration of the MethSO products were obtained by proton NMR.

DiTyr and its heavy C^{13} standard were synthesized and purified with the procedure by Malencik et al. (1996). Structural confirmation, purity of >95 %, and concentration of the DiTyr products were obtained by proton NMR and HPLC.

CML and CEL, and their heavy deuterium standards were the generous gift of the laboratory of John Baynes and Susan Thorpe, at the University of South Carolina, Columbia, SC, USA.

$1C^{13}$ L-tyrosine, $6C^{13}$ 3NT, and d3L-methionine were purchased from Cambridge Isotope Labs, Andover, MA, USA. MG-H1, 2 and 3 and G-H 1, 2 and 3 structural isomers and their heavy deuterium standards were purchased from Polypeptide Group, Strasbourg, France.

2-Aminoadipic acid and its heavy (deuterated) standard was obtained from CDN Isotopes, Pointe-Claire, Quebec, Canada. Organix UK, Essex, UK supplied light and heavy (C^{13}, N^{15}) 3DG-H standards. The remaining light and heavy standards were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Chromatographic separations were achieved utilizing an Agilent 1200 series HPLC equipped with a Synergy-Hydro-RP, 4 µM, 250 × 2.0 mm column supplied by Phenomenex (Torrance, CA, USA). Gradient elution was utilized with an ion-pairing agent buffer $A = 20$ mM HFBA in water and $B = 20$ mM HFBA in methanol. At a flow rate of 0.25 ml/min, the following gradient profile was implemented:

Time (mins)	0	3	6	25	35	45	50	70
%B	0	0	13	29	100	100	0	0

Resolution of analytes

Except for the co-elution of a 3DG-H isomer and CEL, baseline resolutions were achieved for the remaining compounds.

The Agilent 6410 MS/MS, equipped with an ESI source was operating in the positive mode under the following conditions: drying gas was at 350 °C with a flowrate of 10 L/min. Nebulizer pressure was 40 psi and capillary voltage was set for 4,000 for all compounds. Detailed compound analytical parameters are shown in Table 2.

Using isotopic dilution analysis, quantitation of samples was achieved by reading from calibration curves derived from relative response vs relative concentration to the heavy standard. Heavy standards were added to plasma or urine filtrates at final concentrations from 1 to 6 μM in concordance with the expected physiological concentrations and range of the standard curves.

The order of elution of the compounds is as shown in Table 3. This table also shows the coefficient of variation (COV) for repeated between day measurements of the analytes, as well as the lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) for each analyte.

Analysis of urinary AGEs and oxidation products to determine the degree of variability

We initially performed studies to determine if urinary levels of oxidation products (MethSO and aminoadipic acid) showed evidence of artifactual production. For these studies, we analyzed four urine filtrates per subject (208 samples in 52 subjects) from a large NHS subgroup. Based on our observation that between subject variability of MethSO and all other biomarkers significantly exceeds the within subject variability, we concluded that there was no evidence of oxidation artifacts, and that measuring AGEs and OPs in four samples from each subject over 5 years provides optimal representation for each product.

Within analysis variation

For the plasma filtrate analyses, the within analyte COVs for ten consecutive runs for the same plasma filtrate varied from 8.6 (MGHI) to 11.6 % for G-HI.

Statistical analyses

Both continuous and categorical statistical methods were used to investigate the relationships between the AGEs/OPs and nephropathy progression based on 5 years change in GBM width and mesangial fractional volume.

Table 2 Compound analytical parameters for mass spectrometry

Compound[a]	Transition	Fragmentor V	Collision V
CML (Quantifier)[b]	205.1–84.1	100	22
CML (Qualifier)[b]	205.1–130.1	100	8
d4 CML (Isotope)[b]	209.1–88.1	100	22
CEL	219.1–84.1	100	22
CEL	219.1–130.1	100	8
d4 CEL	223.1–88.1	100	22
MethSO	166.1–74.1	80	7
MethSO	166.1–102.1	80	10
d3 MethSO	169.1–74.1	80	7
3-NT	227–181	94	8
3-NT	227–117	94	20
$6C^{13}$ 3-NT	233–187	94	8
MG-H1	229–166.1	104	13
MG-H1	229–114	87	12
d3 MG-H1	232.1–169.2	104	13
MG-H2	229–116	87	12
MG-H2	229–114	87	12
d3 MG-H1	232.1–169.2	104	13
MG-H3	229–114.1	87	12
MG-H3	229–116	87	12
d3 MG-H1	232.1–169.2	104	13
DiTyr	361.1–315.1	100	10
DiTyr	361.1–254.1	100	18
$2C^{13}$ DiTyr	363.1–316.1	118	12
G-H1	215–152	80	9
G-H1	215–116	80	5
$2C^{13}$ G-H1	217.2–154.1	104	12
G-H2	215.1–116.1	80	5
G-H2	215.1–100.1	100	10
$2C^{13}$ G-H1	217.2–154.1	104	12
G-H3	215.1–100.1	100	10
G-H3	215.1–116.1	80	5
$2C^{13}$ G-H1	217.2–154.1	104	12
3DG-H	319.1–204.2	120	14
3DG-H	319.1–116.1	120	22
$6C^{13}4\ N^{15}$ 3DG-H	329.1–208.1	120	14
AAA	162.2–98.1	52	12
AAA	162.2–144.2	52	4
d3 AAA	165.2–101.2	55	12

[a] Capillary voltage was set at 4,000 for all transitions

[b] Transitions remain in the same order throughout table

Linear regression analyses that include each biomarker as a continuous covariate were fit to data from 103 participants. Both simple linear and multiple linear regression analyses were performed, with the multiple regression covariates including the last HbA1c, diabetes duration, and gender when analyzing GBM, and age added to this list for

Table 3 Validation of analytical method

Compound	Mean (nM)[a]	Between day COV (%)[a]	LLOD[b] (nM)	LLOQ[b] (nM)
MethSO	1,610	11.2	64	210
AAA	1,380	9.6	81	270
CML	110	10.1	10	34
CEL	62	10	8.6	28
3DG-H	450	10.9	40	130
G-H1	22	11.6	3.1	10
MG-H1	303	8.6	5.8	19
3-NT	<LLOQ	–	2.2	7.1
DiTyr	<LLOQ	–	2.9	9.5

R^2 value exceeds 0.99 for all calibration curves

[a] Calculated from replicate injections of a pooled plasma filtrate, $n = 18$

[b] Calculated from the standard deviation of the response (SD) and the slope (S) of calibration curves. LLOD = 3.3 (SD/S), LLOQ = 10 (SD/S) values represent the mean of five calibration curves

analyses of change in mesangial expansion score (MES). MES was defined as the change in mesangial fractional volume factored for the time between baseline and follow-up biopsies. Wilcoxon rank-sum (nonparametric) analyses were used to compare levels of AGEs and OPs in fast-slow DN progressor groups.

Using logistic regression, we also developed predictive equations relating each biomarker in plasma filtrates to the probability of classification as "fast" progression of nephropathy. CML and MGH1 values were log-transformed when used as predictors. We also determined the odds ratio for a 1 standard deviation difference in CML, CEL, and MGHI, as well as HbA1c. Forest plots were used to display the estimate odds ratios (and 95 % CI) for a 1 standard deviation difference in CML, CEL, and MGHI.

Results

Initial analysis of serum

Serum samples were initially evaluated for quality control purposes from 10 of 103 randomly chosen study subjects where 4–6 samples were available for analysis ($n = 50$) over the 5 years of the "NHS". As shown in Fig. 1, the serum filtrates (SF) showed that MethSO was elevated in some serum filtrate samples by 10–20-fold relative to the levels that we have consistently observed in the past in biological samples, and that this striking variability occurred in samples at apparently random time points from the same research subjects.

To determine if similar artifactual changes were seen in plasma, we analyzed MethSO in filtrates from the same subjects used for determination of serum filtrates. These samples were collected 10 years ago by a rigorous protocol and stored at −80 °C in our laboratory. No evidence of artifactual oxidation was seen in these plasma filtrates relative to serum filtrates (mean 1,100 vs. 16,100 nM for plasma and serum, respectively), with the plasma levels being similar to six diabetic subjects from which fresh plasma was processed and analyzed (mean 1,710 nM). These results confirmed that careful preparation could prevent artifactual protein oxidation.

Analysis of relationship of urinary biomarker levels with DN in full population of nephropathy progressors and non-progressors

As shown in Table 4, only urinary levels of CEL ($p = 0.039$) showed a significant difference between groups. CML ($p = 0.10$) and DiTyr ($p = 0.16$) were borderline significant, with all other p values >0.25. A non-parametric method (Wilcoxon rank-sum test), also confirmed that CEL ($p = 0.02$) was different between groups. No biomarker correlated with the degree of mesangial expansion over 5 years. When adjusted for other covariates (HbA1c, age, and duration of diabetes), however, urinary CEL was no longer significantly elevated in FP with p values changing from 0.039 for the univariate to 0.82 for the multivariate analysis and Beta changing from 1.17 to −0.012, respectively. These outcomes suggest that multiple urinary AGEs, and Oxidative biomarkers did not show a statistically significant independent relationship with progression of biopsy proven diabetic nephropathy.

Stability of AGEs and OPs in plasma filtrates over time with storage

We initially analyzed the mean levels of our AGEs and OPs in filtrates of NHS plasma samples stored at −80 °C

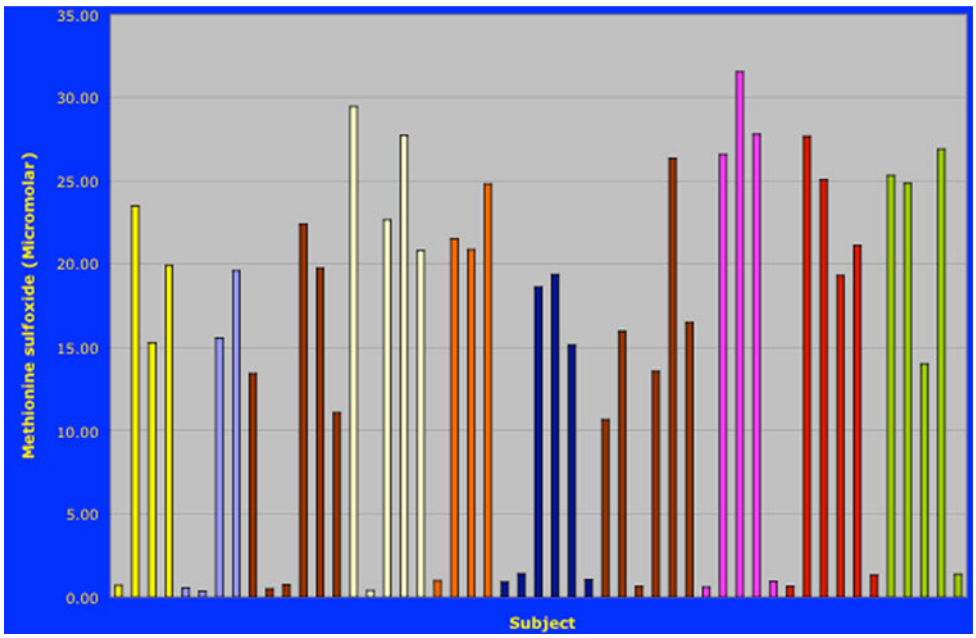

Fig. 1 Individual levels of MethSO in serum filtrates are shown for each of the ten subjects initially screened for quality control. The 4–6 samples analyzed for each subject is color coded, and each *vertical bar* represents an separate sample. The MethSO values show a biphasic distribution, with the higher, artifactually produced values increasing 10–20-fold

Table 4 Urinary biomarker levels in nephropathy progressors and non-progressors

	n	CML	CEL	MG-HI	3DG-H	GH-I	DiTyr	MethSO	AAA
Progressor	35	15.1 ± 5.4	7.29 ± 1.83	41.4 ± 17.6	51.2 ± 18.5	1.35 ± 0.63	0.058 ± 0.012	5.54 ± 4.09	96.9 ± 46.9
Non-progressor	89	13.3 ± 5.5	6.46 ± 2.05	38.7 ± 19.3	47.5 ± 21.9	1.30 ± 0.47	0.055 ± 0.013	5.09 ± 3.36	93.8 ± 53.5
p value		0.097	0.015	0.32	0.16	0.99	0.076	0.76	0.67

Expressed as μ mols/g creatinine

for 10 years, and compared these results with freshly drawn and prepared plasma samples from six young subjects with T1DM. These analyses, even for products sensitive to oxidative stress, such as MethSO, showed similar mean levels of both AGEs and OPs (data not shown).

Three AGE biomarkers from plasma filtrates were significantly higher in fast progressors

As shown in Table 5, MGHI, CEL, and CML levels were significantly higher in GBM-defined FP relative to SP. The other AGE and oxidative biomarkers measured (GHI, 3DGHI, MethSO, and AAA) were not associated with DN progression (data not shown). Levels of 3-NT and dityrosine were below limits of detection. No AGE or OP predicted mesangial expansion.

Three AGEs in plasma filtrates add to predictive value of HbA1c

Further regression analyses exploring the value of these three biomarkers in predicting early DN found that HbA1c at year 5 accounted for 4.7 % of the variation in GBM width (R^2), but the proportion of variation in GBM width accounted for was increased to 11.6 % when MGHI, CEL, and CML were added to the model (6.9 % increase). Thus, these three AGEs increase (over HbA1c) the amount of variation in GBM width identified in the regression analysis. While controlling for HbA1c, CEL, and CML, MGHI was a significant independent predictor of prior GBM increase; MGH1 accounts for an increase of 4.2 % in the R^2 compared to a model containing HbA1c, CEL, and CML.

We used logistic regression models to develop predictive equations relating each biomarker to the probability of a subject's classification as a "fast" progression of DN. CML and MGH1 values were log-transformed when used as predictors and then back-transformed when creating predictive probability plots. For the three biomarkers, but not for HbA1c ($p = 0.28$) measured at the same time, there was a significant relationship to the probability of classification as a FP (CML $p = 0.02$; CEL $p = 0.03$; MGHI $p = 0.048$). For HbA1c, the relationship was only significant when fit to the entire sample ($n = 186$) over 5 years ($p = 0.006$).

We observed that the odds ratios (and 95 % CI) for a one standard deviation change in the three informative biomarkers (CML, CEL, and MGHI), and HbA1c, as

Table 5 Three significant plasma "free fraction" biomarker levels in nephropathy progressors and non-progressors: based on rates of GBM thickening over 5 years

Biomarker (all nM)	Fast (mean ± SD)	Slow (mean ± SD)	p value Wilcoxon rank sum	Number progressors/non-progressors
CML	88 ± 22	75 ± 23	0.003	24/79
MGHI	200 ± 99	165 ± 127	0.04	24/79
CEL	58 ± 15	49 ± 15	0.026	24/79

Table 6 AGEs and OPs in plasma digests do not predict diabetic nephropathy

Biomarker	t test	Wilcoxon rank-sum
CML	0.62	0.75
MethSO	0.28	0.36
3DG-H	0.61	0.50
CEL	0.70	0.61
MG-HI	0.22	0.30

p values shown for t test and Wilcoxon rank analysis

calculated from the logistic regression model were 1.95, 1.72, 1.68, and 1.28, respectively. For example, a 1 SD increase in CEL would lead to a 1.72 increase in the odds of being in the fast progression group.

AGEs and OP from proteolytic digests of plasma samples did not predict DN

None of the protein-bound AGE or OP biomarkers were related to nephropathy progression or non-progression, based on the degree of GBM or MES change, in single variable or multivariate analysis (covariates of HbA1c, duration of diabetes in both models, with gender in the GBM model and age in the MES model—results not shown). Additionally, MGH1 ($p = 0.30$), MethSO ($p = 0.36$), 3DGHI ($p = 0.50$), CEL ($p = 0.61$), and CML ($p = 0.75$) had no significant association with nephropathy progression when the subjects were grouped by slow/fast progression status (Table 6).

Discussion

We observed significant associations between CEL, CML, and MGHI only when we measured our biomarkers in the plasma 10 k filtrate. Our findings that three specific plasma "free fraction" biomarker levels are significantly higher in biopsy documented DN FP than in SP also documented that these AGEs serve as biomarkers for early, clinical not yet evident DN and add to information provided by HbA1c. More specifically, HbA1c at year 5 accounted for 4.7 % of the variation in GBM width (R^2), while the explained percentage of variation in GBM width was increased to 11.6 % when MGHI, CEL, and CML were added to the model (6.9 % increase) (Roper and McIlwain 1948). The value of these of these three biomarkers for early, clinical not yet evident DN in plasma filtrates was further explored by using a logistic regression model, where equations were developed relating each biomarker to the probability of classification as FP of DN defined as the upper tertile of the rate of increase in GBM width. For example, for CEL a value of 0.3 nM "fast" progression risk of DN was 10 %, while a value of 0.9 nM is associated with a 50 % risk. Using this logistic regression model, we also determined the odds ratio for a one standard deviation difference in the three biomarkers of interest, as well as the HbA1c. These odds ratios for "fast" DN progression for CEL (1.72), MGHI (1.68), and CML (1.95) were statistically significant, while this was not the case for HbA1c (OR = 1.29). Using this logistic regression model, we have shown that CEL, MGHI, and CML are indicators of "fast" DN progression for early, clinical not yet evident DN, while HbA1c is not when done concomitantly.

It is important to note that measurement of multiple AGE and oxidative biomarkers in urine filtrates and in protein-bound products measured on extensive plasma digests did not demonstrate significant associations with progression of DN, while the plasma "free adducts" of three specific biomarkers were indicators of early, clinical not yet evident DN. It is known that cells maintain the quality and functional integrity of proteins by degradation and replacement of damaged proteins, and that oxidation and glycation are major types of protein damage physiologically. (Goldberg 2003; Thornalley et al. 2003) Cellular proteolysis liberates the glycated and oxidized amino acids as free adducts, and these are released into blood plasma and excreted in urine. The changes in plasma concentrations and urinary excretion of glycation and oxidation "free adducts" reflects breakdown products indicating generalized tissue damage in diabetes, and provides markers indicative of the damaging effects of hyperglycemia.(Ahmed et al. 2005a). The different outcomes with plasma "free adducts", relative to AGEs bound to plasma proteins suggests that the fraction reflecting breakdown of damaged proteins more closely reflects the pathophysiologic role of these products than those bound to proteins. The reasons for the discrepancy in

the ability of plasma and urinary "free adducts" as indicators of for early, clinical not yet evident DN is not clear, although it may reflect the highly variable renal clearance of individual AGEs (Ahmed et al. 2005a).

In addition to plasma, serum samples were analyzed accordingly, but suitable data were not obtained, most likely due to improper handling during sampling and storage. This effect was not observed, however, in appropriately handled plasma samples, or urine samples used for this study. Based on these observations, it is likely that plasma is a better choice for measurement of OP and AGEs in stored samples since it contains the trace metal chelating agent (EDTA) and is immediately spun and separated from RBCs and flash frozen after collection. Serum, on the other hand, has to undergo clotting at room temperature before separation and storage, thus exposing proteins to leukocyte myeloperoxidase and other pro-oxidant enzymes. Serum also contains no chelator of trace metals (Fe and Cu), both of which can catalyze spontaneous in vitro oxidative stress.

It should be noted that plasma analyses were done only on a single sample that was collected and processed by a rigorous protocol, while the analysis of urinary biomarkers were performed on four samples spread over the 5 years of the study. Finding correlation of specific AGEs in this single plasma sample with progression of an important DN lesion suggests the possibility that even stronger predictive value could have been obtained with multiple samples collected over a few years. It should also be pointed out that all biomarkers in plasma filtrates were measured at the 5 years point and not at baseline. At this point, the degree of progression or non-progression of DN on the biopsies was apparent, and the accumulation of long-lived AGEs had occurred proportionally. Therefore, "prediction" needs to be qualified since CML, CEL, and MGHI reflected the change in GBM width over the previous 5 years. This does suggest that these three AGEs reflect the long-standing pathogenic processes involved in GBM thickening.

Since two out of three of the predictive biomarkers are end products of methylglyoxal (CEL and MGHI), these outcomes support a role for elevated methylglyoxal levels in the development of DN, and is consistent with increased cellular production of MG in early DN progressors that we have observed (Beisswenger et al. 2005). Since the oxidative end products measured in these studies were not predictive, an association of oxidative stress in DN progression, as proposed by others, was not confirmed (Brownlee 2005).

We used increased GBM width as our primary renal endpoint since increased GBM width was the earliest structural indicator of nephropathy progression to microalbuminuria in an overall NHS study population. Since changes in mesangial fractional volume are, on average, minimal in the first ≈ 15 years of T1DM, it did not serve as an early structural predictor of progression to microalbuminuria (Steinke et al. 2005).

Clearly, we need better predictors of diabetic nephropathy. Microalbuminuria is currently the most commonly used DN risk predictor, but it has been recognized that approximately 40 % of longstanding diabetic patients at risk of DN will have normal urinary albumin when initially screened, there is an imprecise correlation between the degree of biopsy proven DN and the level of MA, and that nephropathy may already be advanced by the time MA develops (Fioretto et al. 1994). Further, only about 30 % of MA T1DM patients will progress to macroalbuminuria. Repeated measures of HbA1c was proven to be useful in predicting diabetic microvascular complications in large outcome studies (DCCT Research Group 1993), but it also has shortcomings as an early predictor of DN. These include its estimation of average blood sugar only over a relatively short 3 months time period, and its inability to fully reflect most of the key chemical pathways that produce complications (Brownlee 2005).

Although these results are compelling with respect to identifying novel biomarkers of progression to DN, future studies would benefit from examining these and additional biomarkers reflecting pathways associated with AGEs in larger study groups. This would enable a clearer ultimate differentiation between fast and slow nephropathy progression. It will also be informative to test the predictive value of these specific AGE biomarkers for diabetic retinopathy and neuropathy utilizing studies where the phenotypes of multiple diabetic complications are documented.

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Conflict of interest The authors declare that they have no conflict of interest.

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