Plasma Proteins Modified by Advanced Glycation End Products (AGEs) Reveal Site-specific Susceptibilities to Glycemic Control in Patients with Type 2 Diabetes

Received for publication, November 11, 2015, and in revised form, February 19, 2016
Published, JBC Papers in Press, March 1, 2016, DOI 10.1074/jbc.M115.702860

Uta Greifenhagen, Andrej Frolov, Matthias Blüher, and Ralf Hoffmann

From the Institute of Bioanalytical Chemistry, Faculty of Chemistry and Mineralogy, the Center for Biotechnology and Biomedicine, and the Department of Medicine, Endocrinology, Universität Leipzig, 04103 Leipzig, Germany

Protein glycation refers to the reversible reaction between aldoses (or ketoses) and amino groups yielding relatively stable Amadori (or Heyns) products. Consecutive oxidative cleavage reactions of these products or the reaction of amino groups with other reactive substances (e.g. α-dicarboxyls) yield advanced glycation end products (AGEs) that can alter the structures and functions of proteins. AGEs have been identified in all organisms, and their contents appear to rise with some diseases, such as diabetes and obesity. Here, we report a pilot study using highly sensitive and specific proteomics approach to identify and quantify AGE modification sites in plasma proteins by reversed phase HPLC mass spectrometry in tryptic plasma digests. In total, 19 AGE modification sites corresponding to 11 proteins were identified in patients with type 2 diabetes mellitus under poor glycemic control. The modification degrees of 15 modification sites did not differ among cohorts of normoglycemic lean or obese and type 2 diabetes mellitus patients under good and poor glycemic control. The contents of two amide-AGEs in human serum albumin and apolipoprotein A-II were significantly higher in patients with poor glycemic control, although the plasma levels of both proteins were similar among all plasma samples. These two modification sites might be useful to predict long term, AGE-related complications in diabetic patients, such as impaired vision, increased arterial stiffness, or decreased kidney function.

The reaction between reducing sugars (i.e. aldoses or ketoses) and amines is termed glycation or nonenzymatic glycosylation (1). The initially formed aldimines or ketimines can rearrange forming relatively stable Amadori or Heyns products. Consecutive rearrangements and oxidations yield advanced glycation end products (AGEs) (2–6), a structurally heterogeneous group of compounds including cross-linked aldoses (or ketoses) and amines is termed glycation or nonenzymatic glycosylation (1). Alternatively, α-dicarboxyls generated by Amadori degradation (7, 8), saccharide autooxidation (9, 10), lipid peroxidation (11, 12), and enzymatic reactions (13–15) can modify the side chains of lysine and arginine residues (16–18). Degradation of glucose-derived Amadori products, for example, yields 1-deoxyglucosone (7, 19) and its tautomers, which upon nucleophilic attack by a Nα-amino group of a lysine residue undergo hydrolytic β-cleavage yielding amide-AGEs, such as Nα-acetyl-, formyl-, and glyceril-lysine (Fig. 1) (20). Generally, AGEs can alter the structure of proteins contributing to diabetic retinopathy (21, 22), increased arterial stiffness (23, 24), or impaired renal function (25) along with aging and diabetes. Furthermore, receptor binding-induced activation of proinflammatory signaling pathways is associated with the development and progression of atherosclerosis (26) and nephropathy (25).

AGEs have been localized in biological tissues by immunohistochemistry (27–29) and quantified with ELISA using monoclonal antibodies recognizing methylglyoxal-derived hydroimidazolone (30), imidazolone (31), pentosidine (32), pyrraline (33), and Nα-carboxymethyl lysine (34, 35). Alternatively, individual modifications were assayed by their intrinsic fluorescent properties (36–38) or after derivatization with a fluorophore (37, 39). AGEs can be detected likewise after chromatographic separation by mass spectrometry without derivatization in the multiple reaction monitoring mode, allowing their quantification with high sensitivity (40, 41) and robustness (42). Characterization of amide-AGEs in proteomes will help with understanding the development of glycation-associated pathologies by identifying the affected protein (function) and providing access to subtissue level changes of individual residues prone to such modifications. We recently reported a two-step procedure to detect AGE-modified residues in plasma samples using specific and sensitive precursor ion scans (43, 44). Here, we extended this approach by using gas phase fractionation to detect amide-bound AGEs that have been previously identified in lens homogenate (41) and as free adducts in human plasma and urine and found to be increased in uremic dialysis patients (45). These peptides were relatively quantified in plasma samples obtained from obese type 2 diabetes mellitus (T2DM) patients with good or poor glycemic control and compared with healthy lean and obese persons.

Experimental Procedures

Materials—Chemicals and materials were from Carl Roth (Karlsruhe, Germany): tris(2-carboxyethyl)phosphine (≥98%), glycerin (≥99.5%); Biosolve (Valkenswaard, Netherlands); ace-
Protein-AGEs as Prospective Glycemic Control Markers in Plasma

FIGURE 1. Structures of N’-glycinyl- (A), acetyl- (B), and formyl-lysine (C). R represents the ε-norleucin rest.

tonitrile (≥99.97%); AppliChem (Darmstadt, Germany): Tris (ultrapure); Serva Electrophoresis (Heidelberg, Germany): acrylamide/bis solution (37.5:1, 30% (w/v), 2.6% C), ammonium persulfate (analytical), N,N,N’,N’-tetramethylethlenediamine (research), Coomassie Brilliant Blue G-250 (pure), porcine trypsin (NB premium grade); Riedel-de-Haën (Seelze, Germany): bromophenole blue (reagent); Objective (Berlin, Germany): cine trypsin (NB premium grade); Riedel-de-Haën (Seelze, Germany): bromophenole blue (reagent); Objective (Berlin, Germany): PicoTip online nano-ESI emitter (standard coating; 3 mm in length, m and tip internal diameter 10 μm); Sartorius Stedim Biotech GmbH (Göttingen, Germany): Vivaspin filter devices (5000 molecular weight cut-off); Sigma-Aldrich (St. Louis, Missouri, USA): 2-mercaptoethanol (≥98%), 2-mercaptoethanol (≥99.0%). Water was purified in house (resistance ≥ 18 mΩ, total organic content ≤ 0.1 ppb) with a PureLab Ultra Analytic System (ELGA Lab Water, Celle, Germany).

**Plasma Samples**—Blood was obtained from male healthy, normoglycemic persons (HbA1c < 5.6%; fasting plasma glucose ≤ 6.0 mmol/liter) categorized as lean (body mass index < 25 kg/m², n = 6) or obese (body mass index > 30 kg/m², n = 5), as well as from obese T2DM patients (HbA1c > 6.5%, fasting plasma glucose > 6.0 mmol/liter) being under poor (HbA1c ≥ 7.5%, n = 5) or good glycemic control (HbA1c = 6–7%, n = 6). Cohorts were matched for age (50 years) and, in case of obese cohorts, for body mass index (supplemental Table S1). All blood samples were collected between 8 and 9 am after a 12-h fast in EDTA-treated tubes, blood cells were removed from plasma by centrifugation for 10 min at 2,000 × g, and the separated plasma was stored at −80 °C. The study was approved by the ethics committee of the University of Leipzig (approval no. 159-12-21052012), and all subjects gave written informed consent before taking part in the study.

**Tryptic Digestion**—Plasma samples (100 μl) were depleted of lipids by centrifugation (9168 × g, 30 min, 4°C), an aliquot (20 μl) diluted 10-fold with ammonium hydrogen carbonate buffer (0.1 mol/liter, pH 8.0), and desalted using ultrafiltration (Vivaspin filter devices, 0.5 ml, 5-kDa cut-off). Protein concentrations were determined by a Bradford assay (46). Briefly, acidic Coomassie Brilliant Blue G-250 solution (0.1 g/liter in 10% (v/v) H₃PO₄, 5% (v/v) aqueous ethanol, 250 μl) was mixed with the protein sample (5 μl) in a 96-well microtiter plate, and the absorption was recorded in two replicates at 595 nm. Quantification relied on a 2-fold dilution series of BSA (1000 to 62.5 μg/liter). Plasma aliquots corresponding to a protein content of 25 μg were complemented with SDS (10% (w/v)) in water, 2 μl and tris(2-carboxyethyl)phosphine (50 mmol/liter in water, 2 μl), diluted with aqueous ammonium hydrogen carbonate buffer (50 mmol/liter, pH 8.0) to obtain a final volume of 20 μl, and incubated at 60 °C for 15 min. The samples were cooled to room temperature, alkylated with iodoacetamide (0.1 mol/liter, 2.2 μl) in the darkness (15 min, room temperature), and digested with trypsin (25 mg/liter in 50 mmol/liter ammonium hydrogen carbonate, 50 μl, 37 °C, overnight). The digest was frozen and stored at −80 °C. An aliquot (2 μg protein) of the sample was diluted with sample buffer (0.05% (w/v) bromphenol blue, 62.5 mmol/liter Tris-HCl, pH 6.8, 20% (v/v) glycerin, 2% (v/v) SDS, 5% (v/v) β-mercaptoethanol) at least 2-fold and heated to 95 °C for 5 min. Proteins were separated by SDS-PAGE (T = 12%, C = 2.65%) (47) and stained with colloidal Coomassie Brilliant Blue G 250 (48). The digestion step was considered complete (>99%) when the original HSA band was not detected.

**Nano UPLC-ESI-Linear Ion Trap-Orbitrap-MS**—Plasma samples (75 ng) were loaded on a nanoAcquity UPLC Symmetry™ trap column using a flow rate of 5 μl/min for 5 min and separated on a nanoAcquity UPLC BEH130™ column (30°C) using a nanoAcquity™ UPLC System equipped with an Acquity sample manager (10 μl of injection volume, full loop injection) and a nanoAcquity UPLC binary solvent manager (Waters, Eschborn, Germany). Eluents A and B were water and acetonitrile, respectively, both containing formic acid (0.1% v/v). The peptides were eluted with a linear, two-step gradient (3 → 50% eluent B in 45 min, 50 → 85% eluent B in 2 min) at a flow rate of 0.4 μl/min. The column was connected via a PicoTip online nano-ESI emitter to a nanoESI-Orbitrap-MS (LTQ Orbitrap XL ETD) operated in positive ion mode and controlled by Xcalibur 2.0.7 software (Thermo Fisher Scientific, Bremen, Germany). Mass spectra were acquired in the Orbitrap at a resolution of 60,000. Tandem mass spectra (MS/MS) were recorded for the six most intense signals (z ≥ 2) of an Orbitrap survey scan by data-dependent acquisition in the linear ion trap. Tandem mass spectra were analyzed with Sequest against the SwissProt database using Proteome Discoverer 1.1.0.263 (Thermo Fisher Scientific). Amide-AGE-modified peptides identified with an Xcorr below 2.20 for doubly and 3.75 for triply protonated ions were manually confirmed (49). Relative, label-free quantification relied on the integration of extracted ion chromatograms (m/z ± 0.02) for each of the three technical replicates acquired per sample (supplemental Table S2).

**Statistics**—Cohort comparison, statistical calculations, and graphical display were performed with the software GraphPad Prism 5.02 testing the technical replicates’ averages within a cohort first for normal distribution (Kolmogorov-Smirnov test, α = 0.05). If both cohorts to be compared showed normal distribution, a t test was performed to assess the significance of differences. Otherwise, the nonparametric Mann-Whitney test was used (both two-sided, α = 0.05). Correlation analysis was performed using linear regression.

**Results**

**Detection of Amide-AGEs in Diabetic Plasma**—A pooled tryptic plasma digest obtained from five poorly controlled T2DM patients was analyzed repeatedly by nano-reversed phase-UPLC-ESI-linear ion trap-Orbitrap-MS in gas phase fractionation mode by splitting the m/z range from 400 to 1400 in 18 consecutive intervals. When the tandem mass spectra acquired in data-dependent acquisition mode were searched for amide-AGE-modified peptides, 2 glycerinylated, 7 acety-
lated, and 10 formylated peptides were identified corresponding to 11 proteins (Table 1) within the 2626 peptides identified with high and medium confidence by Sequest. For example, peptide SK$_{\text{formyl}}$EQLTPLIK (m/z 592.8457, z = 2) corresponding to apolipoprotein A-II 68–77 (processed: 50–59) eluted at 25.6 min, whereas further isobaric peptides IQNILTEEPK (m/z 592.8289, z = 2, serum paraoxonase/arylersterase 1) and DVE-DAHSSGLIK (first $^{13}$C-isotope of m/z 592.3051, z = 2, N-acetylglucosamine-1-phosphotransferase subunits $\alpha/\beta$) eluted at 24.1 and 26.6 min, respectively (Fig. 2).

Quantification of Amide-AGEs in Plasma—Seventeen of the identified amide-AGE-modified peptides could be quantified by the peak areas displayed in the extracted ion chromatograms in all plasma samples with relative standard deviations except for DRQC$_{\text{diox}}$K$_{\text{acetyl}}$YIW$_{\text{ox}}$GQK, which was not detected in samples 17, 19, and 22. Peptides EFAK$_{\text{acetyl}}$EIDCSVK and LK$_{\text{acetyl}}$C$_{\text{acyl}}$DEW$_{\text{diox}}$SVNVGK were detected in all or only seven samples, respectively, but always too weak for quantification (signal to noise ratio < 10). The physiological state of the subjects (nornormal glycemic lean or obese, well or poorly controlled T2DM) did not affect the quantities of 15 of the 17 peptides ($p > 0.05$, average differences < 50%; supplemental Fig. S1). In contrast, LK$_{\text{acetyl}}$C$_{\text{cam}}$ASLQK (HSA 222–229, processed: 198–205) allowed distinguishing poorly controlled diabetic patients from lean healthy persons ($p = 0.017$, without highest data point: $p = 0.038$; Fig. 3A). Moreover, SK$_{\text{formyl}}$EQLTPLIK from apolipoprotein A-II (Fig. 3B) was able to discriminate poorly controlled patients with T2DM from all other cohorts (all $p < 0.002$). To ensure that these higher levels in poorly controlled patients with T2DM were not associated with an elevated level of the protein (i.e. similar modification rate, but higher protein quantities), nonmodified peptides corresponding to both proteins were quantified as well. HSA quantification relied on 21 non-AGE-modified peptides identified with high and one peptide (LK$_{\text{cam}}$ASLQK) with medium confidence scores (49) (all peptides ranked at position 1), whereas five peptides of medium confidence were chosen for apolipoprotein A-II (supplemental Table S3). Because the plasma levels of both proteins (assessed via the majority of the non-AGE-modified peptides; supplemental Fig. S2) did not differ between the groups, the increase in modified peptides can be attributed solely to elevated modification degrees at both sites. The modification rate of LK$_{\text{cam}}$ASLQK (HSA) was even so high that the quantity of the unmodified peptide decreased in the poorly controlled patients with T2DM relative to all other cohorts albeit not significantly ($p > 0.05$; supplemental Fig. S2G).

Most likely, the lower content of the unmodified peptide was not attributed completely to the acetyl-AGE, but to other AGE modifications as well. The obtained differences in the cohort averages from the background of a limited cohort size ($n$) and the observed intracohort standard deviations ($\sigma$) were statistically evaluated for the minimum meaningful difference ($d$). The minimum $n$ required to demonstrate meaningful $d$ with typical confidence levels ($\alpha = 0.05$, $\beta = 0.2$) was calculated with the transposed form of formula $n = 16 \times \sigma^2/d^2$. Indeed, the minimum $d$ calculated for SK$_{\text{formyl}}$EQLTPLIK between T2DM_LT and T2DM_MT was by a factor of 0.75 smaller than the observed differences of the cohort averages.

Correlation analysis with the patients’ clinical data (supplemental Table S1) showed either none or only weak associations to body mass index, plasma protein concentration, or HbA$_1c$ except for SK$_{\text{formyl}}$EQLTPLIK (apolipoprotein A-II), which strongly correlated to HbA$_1c$ ($R^2 = 0.79$; Fig. 4).

Discussion

Detection of Amide-AGEs in Diabetic Plasma—The structures of peptides comprising N$^\alpha$-acylated, formylated, and glycerinated lysine residues can be easily determined by tandem mass spectrometry, because all three modifications are stable against collision-induced dissociation (50). Thus, standard MS-based proteomic approaches can be applied to identify accordingly modified sites. Gas phase fractionation of pooled plasma samples was sufficiently sensitive to identify 19 modified sites in 11 plasma proteins. Most common were acetylation and formylation sites among the identified amide-AGEs, which is in accordance with the ratio of free AGE-modified amino acids in healthy and uremic plasma (45). Other modifications apart from nonenzymatic glycosylation might be enzymatic reactions or acetylating agents (e.g. acetylsalicylic acid). Lys$_{223}^{N\text{-acetyl}}$ is the first site that was identified in HSA to be acetylated by acetylsalicylic acid (51) and was, together with Lys$_{549}^{N\text{-acetyl}}$, detected in “pure” HSA products (52). Additionally, acetylated Lys$_{88}$ was detected in nondiabetic plasma even prior to incubation with acetylsalicylic acid (53), but none of the other sites observed after HSA or plasma incubation with acetylsalicylic acid were found (52, 53). Enzymatic acetylation (and formylation) are regulatory functions in nucleus, liver cell cytosols, and mitochondria (54), but such activities have not been reported to our knowledge in plasma. In contrast, all HSA and apolipoprotein A-II sites identified here as modified by amide-AGEs are known to be glycated in plasma from patients with T2DM (55, 56), indicating that these sites are susceptible to glycation finally leading to AGEs.

Relative Label-free Quantification of Amide-AGE Peptides in Plasma—Fifteen peptides (90%) were present in comparable quantities in all plasma samples indicating a relatively constant basic level of AGEs in human plasma that is not influenced by obesity and diabetes. This view is supported by studies at the amino acid level of plasma protein hydrolysates that detected similar global AGE levels in healthy persons (45, 57).

Most detected AGE modification levels were not influenced by the patients’ glycemic status considering the HbA$_1c$ level. This is probably attributed to the different formation pathways of the N$^\alpha$-(fructosyl) moiety, because hemoglobin is directly modified by glucose, whereas the studied N$^\alpha$-AGEs require first glucose degradation prior to alkylation of lysine residues. Further parameters probably contributing to the lower sensitivity in reflecting the blood glucose level are the different protein localizations (intracellular versus plasma), protein life times, and the site-specific susceptibilities to undergo modification. Thus, N$^\alpha$-AGEs are likely long term markers. Indeed, two amide-AGE modification sites were present at elevated levels in diabetic patients with poor glycemic control, most likely representing highly susceptible residues. Additionally, the generally
| No. | Sequencea | tR  | m/z   | z   | p    | XCorr | Accession no. | Name | Site |
|-----|-----------|-----|-------|-----|------|-------|---------------|------|------|
| 1   | TCcamVADESAENCoxDKacetyl | 13.5 | 750.2850 | 2 | 27.79 | 3.56  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)b |
| 2   | LDELRDEGKacetylASSAK | 18.0 | 530.9321 | 3 | 3.68  | 2.83  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)b |
| 3   | LKacetylASLQK | 19.0 | 740.8950 | 2 | 42.21 | 2.46  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)b |
| 4   | KacetylQTALVELVK | 27.0 | 585.8586 | 2 | 1.23  | 1.79  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)b |
| 5   | DRQCdioxKacetylYIWoxGQK | 22.5 | 757.8620 | 2 | 4.82  | 2.23  | P02768 | Disintegrin and metalloproteinase domain-containing protein 22 (ADAM 22) (ADA22_HUMAN) | Lys555 (Lys530)c |
| 6   | EFAKacetylEIDISCVK | 26.5 | 712.3556 | 2 | 2.17  | 2.60  | P29323 | Ephrin type-B receptor2 (EPHB2_HUMAN) | Lys68 (Lys64)d |
| 7   | P6KacetylAEQVSVSK | 21.8 | 777.8638 | 2 | 5.40  | 2.50  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 8   | LFcamKYHELQVQK | 27.2 | 627.8010 | 2 | 1.00  | 1.85  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 9   | KacetylLYLEIAR | 27.1 | 542.2947 | 2 | 41.89 | 1.97  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 10  | YTCcamRNQDISSK | 18.0 | 736.3198 | 2 | 27.79 | 2.46  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 11  | LFcamQKacetylERPLEK | 22.3 | 787.8982 | 2 | 12.50 | 2.53  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 12  | KacetylQTLVELVK | 26.7 | 578.8066 | 2 | 1.23  | 1.79  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 13  | KacetylECcamCcamEKPLLEK | 22.3 | 787.8982 | 2 | 12.50 | 2.53  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 14  | KacetylQTALVELVK | 26.7 | 578.8066 | 2 | 1.23  | 1.79  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 15  | KacetylQTLVELVK | 26.7 | 578.8066 | 2 | 1.23  | 1.79  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 16  | FSGSGSGTDFTLKformyl | 24.4 | 666.3157 | 2 | 45.28 | 2.90  | P06309 | Ig kappa chain V-II region GM607 (LysV205_HUMAN) | Lys68 (Lys64)f |
| 17  | NTLYLQMSNLKformyl | 28.1 | 676.8516 | 2 | 19.45 | 2.48  | Q53H26 | Transferrin variant (Q53H26_HUMAN) | Lys68 (Lys64)d |
| 18  | KformylEQLTPLIK | 25.6 | 592.8457 | 2 | 31.58 | 2.10  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 19  | AEDTAVYYCcamAKformyl | 20.2 | 659.7896 | 2 | 55.07 | 3.22  | Q9Y509 | VH3 protein (Q9Y509_HUMAN) | Lys68 (Lys64)d |
| 20  | AEDTAVYYCcamAKformyl | 21.2 | 673.8044 | 2 | 42.41 | 2.58  | A2IPF7; A2NUT3; A2NKM6 | A2IPF7_HUMAN; Mu-chain (A2NUT3_HUMAN); NANUC-1 heavy chain (A2NKM6_HUMAN) | Lys68 (Lys64)d |
| 21  | VEQDNLKacetyl | 19.2 | 518.7520 | 2 | 1.23  | 1.79  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 22  | FSCGSGSGTDFTLKformyl | 20.2 | 673.8044 | 2 | 42.41 | 2.58  | VN139 | VH3 protein (Q9Y509_HUMAN) | Lys68 (Lys64)d |
| 23  | KformylEQLTPLIK | 25.6 | 592.8457 | 2 | 31.58 | 2.10  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |
| 24  | KformylEQLTPLIK | 25.6 | 592.8457 | 2 | 31.58 | 2.10  | P02768 | Serum albumin (ALBU_HUMAN) | Lys68 (Lys64)d |

a Ccam, carbamidomethylated cysteine; Ccam/Wdiox, oxidized cysteine/tryptophan; Kacetyl, acetylated lysine; Kglycerinyl, glycerinylated lysine.
b Numbering for processed protein, i.e. lacking residues 1–24 corresponding to the N-terminal signaling peptide.
c Numbering for processed protein, i.e. lacking residues 1–25 corresponding to the N-terminal signaling peptide.
d Numbering for processed protein, i.e. lacking residues 1–18 corresponding to the N-terminal signaling peptide.
e Numbering for processed protein, i.e. lacking residues 1–19 corresponding to the N-terminal signaling peptide.
f Numbering for processed protein, i.e. lacking residues 1–4 corresponding to the N-terminal signaling peptide.
g Numbering for processed protein, i.e. lacking the N-terminal starting methionine.
poor correlation of the modification degrees among different sites within one protein (data not shown) emphasizes the site-specific susceptibilities. This might be attributed to nearby residues or more generally the local environment, i.e. reagent accessibility and nearby functional groups affecting the $pK_a$ of the Lys residue or acting as local acid/base catalysts in glycation reactions (56, 58, 59). Furthermore, the differences were relatively moderate (3–6-fold compared with the other cohorts) and were detected only in one peptide per modification type, which suggests that the changes would not be detectable with an analytical approach relying on protein hydrolysis. Hence, AGE modification sites appear to be highly relevant as biomarkers and to understand the pathology of diabetes.

Compared with the glycemically poorly controlled patients with T2DM, the well controlled cohort showed $SK_{formyl}$-EQLTPLIK levels comparable with healthy controls (Fig. 3B), illustrating on the molecular level that glycemic control does not only reduce protein glycation levels but also prevents elevated advanced glycation degrees at sites prone to AGE modifications. Thereby, the T2DM-associated risk to develop AGE-related severe complications can be reduced, which has been previously supported by clinical studies (60). The two amide-AGE modification sites identified here closely resemble poorly controlled patients with T2DM and thus might be helpful to monitor the risk of late complications in diabetes. However, the three individuals showing much higher $LK_{acetyl-CamASLQK}$ levels compared with all other participants (patients 3, 8, and 14), underwent prescribed acetylsalicylic acid treatment, which most likely caused the modification increase and thus may impact its biomarker value.

In conclusion, we demonstrate that bottom-up MS-based proteomics is sufficiently sensitive to detect protein-bound amide-AGE modifications in human plasma without the need for protein hydrolysis. This platform allows direct analysis of human plasma samples and provides new insights into the glycemic control monitoring and the risk of diabetic complications.
for AGE enrichment strategies, which simplifies the analysis and improves the robustness. Because of the site-specific response to the glycemic status in patients, which were most likely subissue level changes, we conclude that site-specific detection and quantification of AGES may prove very useful in AGE-related biomarker discovery and pathology research. However, validation of these results in larger cohorts and with more robust quantification methods (e.g., LC–multiple reaction monitoring) is required in future. Those studies could probably reveal whether and which AGE sites could be suitable as biomarkers reflecting the disease severity in a graded manner, which could be useful to trace or even predict disease progression toward severe complications. As further amide-AGE sites were detected, albeit at too low confidence, further fractionation via orthogonal separation techniques like hydrophilic interaction chromatography might extend the range of detectable modification sites even further.

Author Contributions—M. B. designed the cohorts, provided all plasma samples, and contributed to data interpretation. A. F. acquired the LC–MS and contributed to everything. U. G. conducted data analysis and interpretation and wrote the manuscript together with R. H. R. H. planned the studies, received funding, contributed to study design and data interpretation, and wrote the manuscript together with U. G. All authors reviewed the results and approved the final version of the manuscript.

References

1. Ulrich, P., and Cerami, A. (2001) Protein glycation, diabetes, and aging. Recent Prog. Horm. Res. 56, 1–21
2. Ahmed, M. U., Thorpe, S. R., and Baynes, J. W. (1986) Identification of N\(^\alpha\)-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. J. Biol. Chem. 261, 4889–4894
3. Smith, P. R., and Thornalley, P. J. (1992) Mechanism of the degradation of nonenzymatically glycated proteins under physiological conditions: studies with the model fructosamine, N\(^\alpha\)-(1-deoxy-d-fructos-1-yl)hippuryllysine. Eur. J. Biochem. 210, 729–739
4. Jakas, A., Vinković, M., Smrecki, V., Sporec, M., and Horvat, S. (2008) Fructose-induced N-terminal glycation of enkephalins and related peptides. J. Pept. Sci. 14, 936–945
5. Akira, K., and Hashimoto, T. (2005) Use of C-13 labeling and NMR spectroscopy for the investigation of degradation pathways of Amadori compounds. Biol. Pharm. Bull. 28, 344–348
6. Chetyrkin, S. V., Mathis, M. E., Ham, A. J., Hachey, D. L., Hudson, B. G., and Voynay, P. A. (2008) Propagation of protein glycation damage involves modification of tryptophan residues via reactive oxygen species: inhibition by pyridoxamine. Free Radic. Biol. Med. 44, 1276–1285
7. Hirsch, J., Mossine, V. V., and Feather, M. S. (1995) The detection of some dicarbonyl intermediates arising from the degradation of Amadori compounds (the Maillard reaction). Carbohydr. Res. 273, 171–177
8. Zyzak, D. V., Richardson, J. M., Thorpe, S. R., and Baynes, J. W. (1995) Formation of reactive intermediates from Amadori compounds under physiological conditions. Arch. Biochem. Biophys. 316, 547–554
9. Thornalley, P., Wolff, S., Crabbe, J., and Stern, A. (1984) The autoxidation of glyceraldehyde and other simple monosaccharides under physiological conditions catalyzed by buffer ions. Biochim. Biophys. Acta 797, 276–287
10. Wolff, S. P., and Dean, R. T. (1987) Glucose autoxidation and protein modification: the potential role of autodioxidative glycosylation in diabetes. Biochem. J. 245, 243–250
11. Niyati-Shirkhodaei, F., and Shibamoto, T. (1993) Gas chromatographic analysis of glyoxal and methylglyoxal formed from lipids and related compounds upon ultraviolet-irradiation. J. Agric. Food Chem. 41, 227–230
12. Loidl-Stahlhofen, A., and Spitteler, G. (1994) \(\alpha\)-Hydroxyaldehydehyd, products of lipid peroxidation. Biochim. Biophys. Acta 1211, 156–160
13. Schalkwijk, C. G., Stehouwer, C. D., and van Hinsbergh, V. W. (2004) Fructose-mediated non-enzymatic glycation: sweet coupling or bad modification. Diabetes Metab. Res. Rev. 20, 369–382
14. Phillips, S. A., and Thornalley, P. L. (1993) The formation of methylglyoxal from triose phosphates: investigation using a specific assay for methylglyoxal. Eur. J. Biochem. 212, 101–105
15. Ray, M., and Ray, S. (1987) Aminoacetone oxidase from goat liver: formation of methylglyoxal from aminoacetone. J. Biol. Chem. 262, 5974–5977
16. Glomb, M. A., and Pfahler, C. (2001) Amides are novel protein modifications formed by physiological sugars. J. Biol. Chem. 276, 41638–41647
17. Ahmed, N., and Thornalley, P. J. (2002) Chromatographic assay of glycation adducts in human serum albumin glycated in vitro by derivatization with \(6\)-aminoquinulinyl-N-hydroxyssuccinimidyl-carbamate and intrinsic fluorescence. Biochem. J. 364, 15–24
18. Hasenkopf, K., Rünnser, B., Hilsler, H., and Pischetsrieder, M. (2002) Analysis of glycated and ascorbylated proteins by gas chromatography-mass spectrometry. J. Agric. Food Chem. 50, 5697–5703
19. Gobert, J., and Glomb, M. A. (2009) Degradation of glucose: reinvestigation of reactive \(\alpha\)-dicarbonyl compounds. J. Agric. Food Chem. 57, 8591–8597
20. Smuda, M., Voigt, M., and Glomb, M. A. (2010) Degradation of 1-deoxy-d-erythro-hexo-2,3-diulose in the presence of lysine leads to formation of carboxylic acid amides. J. Agric. Food Chem. 58, 6458–6464
21. Bron, A. J., Sparrow, J., Brown, N. A., Harding, J. J., and Blaktyuty, R. (1993) The lens in diabetes. Eye 7, 260–275
22. Stitt, A. W. (2010) AGEs and diabetic retinopathy. Invest. Ophthalmol. Vis. Sci. 51, 4867–4874
23. Sell, D. R., and Monnier, V. M. (2012) Molecular basis of arterial stiffening: role of glycation: a mini-review. Gerontology 58, 227–237
24. Ziemian, S. J., Melenovsky, V., and Kass, D. A. (2005) Mechanisms, pathophysiology, and therapy of arterial stiffness. Arterioscler. Thromb. Vasc. Biol. 25, 932–943
25. Yamagishi, S., and Matsui, T. (2010) Advanced glycation end products, oxidative stress and diabetic nephropathy. Oxid. Med. Cell. Longev. 3, 101–108
26. Basta, G., Schmidt, A. M., and De Caterina, R. (2004) Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc. Res. 63, 582–592
27. Yan, S. D., Chen, X., Schmidt, A. M., Brett, J., Godman, G., Zou, Y. S., Scott, C. W., Caputo, C., Frappier, T., Smith, M. A., Perry, G., Yen, S. H., and Stern, D. (1994) Glycated Tau-protein in Alzheimer disease: a mechanism for induction of oxidant stress. Proc. Natl. Acad. Sci. U.S.A. 91, 7778–7791
28. Horie, K., Miyata, T., Maeda, K., Miyata, S., Sugiyama, S., Sakai, H., van Ypersole de Strihou, C., Monnier, V. M., Witzum, J. L., and Kurokawa, K. (1997) Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions: implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. J. Clin. Invest. 100, 2995–3004
29. Uchida, K., Khor, O. T., Oya, T., Osawa, T., Yasuda, Y., and Miyata, T. (1997) Protein modification by a Maillard reaction intermediate methylglyoxal: immunological detection of fluorescent 5-methylimidazolone derivatives in vivo. FEBS Lett. 410, 313–318
30. Kilhovd, B. K., Giardino, I., Torjesen, P. A., Birkeland, K., Berg, T. J., Thornalley, P. J., Brownlee, M., and Hanssen, K. F. (2003) Increased serum levels of the specific AGE compound methylglyoxal-derived hydromydizalone in patients with type 2 diabetes. Metabolism 52, 163–167
31. Niwa, T., Katsuizaki, T., Miyazaki, S., Miyazaki, T., Ishizaki, Y., Hayase, F., Tatami, N., and Takei, Y. (1997) Immunohistochemical detection of imidazolone, a novel advanced glycation end product, in kidneys and aortas of diabetic patients. J. Clin. Invest. 99, 1272–1280
32. Miyazaki, K., Nagai, R., and Horiechi, S. (2002) Cretine plays a direct role as a protein modifier in the formation of a novel advanced glycation end product. J. Biochem. 132, 543–550
33. Miyata, S., and Monnier, V. (1992) Immunohistochemical detection of advanced glycosylation end-products in diabetic tissues using monoclonal antibody to pyrraline. J. Clin. Invest. 89, 1102–1112
34. Koito, W., Araki, T., Horiechi, S., and Nagai, R. (2004) Conventional an-
Protein-AGEs as Prospective Glycemic Control Markers in Plasma

tibody against \(N^\omega-(carboxymethyl)\)lysine (CML) shows cross-reaction to \(N^\omega-(carboxyethyl)\)lysine (CEL); immunochemical quantification of CML with a specific antibody. J. Biochem. 136, 831–837

35. Niwa, T., Miyazaki, S., Katsuizaki, T., Tatemichi, N., Takei, Y., Miyazaki, T., Morita, T., and Hirassawa, Y. (1995) Immunohistochemical detection of advanced glycation end-products in dialysis-related amyloidosis. Kidney Int. 48, 771–778

36. Sell, D. R., and Monnier, V. M. (1989) Structure elucidation of a senescent cross-link from human extracellular-matrix; implication of pentoses in the aging process. J. Biol. Chem. 264, 21597–21602

37. Ahmed, N., Argirov, O. K., Minhas, H. S., Cordeiro, C. A., and Thornalley, P. J. (2003) Assay of advanced glycation endproducts (AGEs): surveying AGEs by chromatographic assay with derivatization by 6-aminooquinolyl-N-hydroxyisuccinimidyl-carbamate and application to \(N^\omega-(carboxymethyl)\)lysine- and \(N^\omega-(1-carboxyethyl)\)lysine-modified albumin. Biochem. J. 364, 1–14

38. Uchiyama, A., Ohishi, T., Takahashi, M., Kushida, K., Inoue, T., Fujie, M., and Horiiuchi, K. (1991) Fluorophores from aging human articular cartilage. J. Biol. Chem. 266, 714–718

39. Glomb, M. A., and Monnier, V. M. (1995) Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. J. Biol. Chem. 270, 10017–10026

40. Babaei-Jadidi, R., Karachalias, N., Ahmed, N., Battah, S., and Thornalley, P. J. (2003) Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamin. Diabetes 52, 2110–2120

41. Smuda, M., Henning, C., Raghavan, C. T., Johar, K., Vasavada, A. R., Nagaraj, R. H., and Glomb, M. A. (2015) Comprehensive analysis of Maillard protein modifications in human lenses: effect of age and cataract. Biochemistry 54, 2500–2507

42. Hull, G. L., Woodside, J. V., Ames, J. M., and Cuskey, G. J. (2013) Validation study to compare effects of processing protocols on measured \(N^\omega-(carboxymethyl)\)lysine and \(N^\omega-(carboxyethyl)\)lysine in blood. J. Clin. Biochem. Nutr. 53, 129–133

43. Greifenhagen, U., Nguyen, V. D., Moschner, J., Giannis, A., Frolov, A., and Hoffmann, R. (2015) Sensitive and site-specific identification of carboxymethylated and carboxyethylated peptides in tryptic digests of proteins and human plasma. J. Proteome Res. 14, 768–777

44. Schmidt, R., Böhm, D., Singer, D., and Frolov, A. (2015) Specific tandem mass spectrometric detection of AGE-modified arginine residues in peptides. J. Mass Spectrom. 50, 613–624

45. Henning, C., Smuda, M., Girnrdt, M., Ulrich, C., and Glomb, M. A. (2011) Molecular basis of Maillard amide-advanced glycation end product (AGE) formation in vivo. J. Biol. Chem. 286, 44350–44356

46. Bradford, M. M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248–254

47. Laemmli, U. K. (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature 227, 680–685

48. Neuhoff, V., Stamm, R., and Eibl, H. (1985) Clear background and highly sensitive protein staining with coomassie blue dyes in polyacrylamide gels: a systematic analysis. Electrophoresis 6, 427–448

49. Wolters, D. A., Washburn, M. P., and Yates, J. R. 3rd (2001) An automated multidimensional protein identification technology for shotgun proteomics. Anal. Chem. 73, 5683–5690

50. Greifenhagen, U., Frolov, A., and Hoffmann, R. (2015) Oxidative degradation of \(N^\omega\)-fructosylamine-substituted peptides in heated aqueous systems. Amino Acids. 47, 1065–1076

51. Walker, J. E. (1976) Lysine residue 199 of human serum albumin is modified by acetylsalicylic acid. FEBS Lett. 66, 173–175

52. Liyasova, M. S., Schopfer, L. M., and Lockridge, O. (2010) Reaction of human albumin with aspirin in vitro: mass spectrometric identification of acetylated lysines 199, 402, 519, and 545. Biochem. Pharmacol. 79, 784–791

53. Finamore, F., Priego-Capote, F., Noll, S., Zuffery, A., Fontana, P., and Sanchez, J. C. (2015) Characterisation of the influences of aspirin-acetylation and glycation on human plasma proteins. J. Proteomics 114, 125–135

54. Choudhary, C., Weinert, B. T., Nishida, Y., Verdin, E., and Mann, M. (2014) The growing landscape of lysine acetylation links metabolism and cell signalling. Nat. Rev. Mol. Cell Biol. 15, 536–550

55. Zhang, Q., Tang, N., Schepmoes, A. A., Phillips, L. S., Smith, R. D., and Metz, T. O. (2008) Proteomic profiling of nonenzymatically glycated proteins in human plasma and erythrocyte membranes. J. Proteome Res. 7, 2025–2032

56. Zhang, Q., Monroe, M. E., Schepmoes, A. A., Clauss, T. R., Gritsenko, M. A., Meng, D., Petyuk, V. A., Smith, R. D., and Metz, T. O. (2011) Comprehensive identification of glycated peptides and their glycation motifs in plasma and erythrocytes of control and diabetic subjects. J. Proteome Res. 10, 3076–3088

57. Thornalley, P. J., Battah, S., Ahmed, N., Karachalias, N., Agalou, S., Babaei-Jadidi, R., and Dawnay, A. (2003) Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. Biochem. J. 375, 581–592

58. Venkatraman, J., Aggarwal, K., and Barlaram, P. (2001) Helical peptide models for protein glycation: proximity effects in catalysis of the Amadori rearrangement. Chem. Biol. 8, 611–625

59. Frolov, A., Blüher, M., and Hoffmann, R. (2014) Glycation sites of human plasma proteins are affected to different extents by hyperglycemic conditions in type 2 diabetes mellitus. Anal. Bioanal. Chem. 406, 5755–5763

60. Ceriello, A., Ihnat, M. A., and Thorpe, J. E. (2009) The "metabolic memory": is more than just tight glucose control necessary to prevent diabetic complications? J. Clin. Endocrinol. Metab. 94, 410–415