Human serum albumin is one of the most abundant plasma proteins that readily undergoes glycation, thus glycated albumin has been suggested as an additional marker for monitoring glycemic status. Hitherto, only Amadori-modified peptides of albumin were quantified. In this study, we report the construction of fragment ion library for Amadori-modified lysine (AML), N(ε)-(carboxymethyl)lysine (CML), and N(ε)-(carboxyethyl)lysine (CEL)-modified peptides of the corresponding synthetically modified albumin using high resolution accurate mass spectrometry (HR/AM). The glycated peptides were manually inspected and validated for their modification. Further, the fragment ion library was used for quantification of glycated peptides of albumin in the context of diabetes. Targeted Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) analysis in pooled plasma samples of control, prediabetes, diabetes, and microalbuminuria, has led to identification and quantification of 13 glycated peptides comprised of four AML, seven CML, and two CEL modifications, representing nine lysine sites of albumin. Five lysine sites namely K549, K438, K490, K88, and K375, were observed to be highly sensitive for glycation modification as their respective m/z showed maximum fold change and had both AML and CML modifications. Thus, peptides involving these lysine sites could be potential novel markers to assess the degree of glycation in diabetes.

Diabetes is a complex metabolic disorder characterized by prolonged hyperglycemia resulting from defects in insulin secretion, insulin action, or both, leading to abnormalities in carbohydrate, fat, and protein metabolism (1). According to the projection by the International Diabetes Foundation, around 592 million people will be affected by diabetes by the year 2040 (2). Diabetes and its associated complications are becoming global public health problems and posing a serious challenge in disease management. Many studies have implicated advanced glycation end products (AGEs) in the development of insulin resistance, as well as in pathogenesis of diabetic complications (3). The levels of AGEs increase substantially in diabetic plasma due to the hyperglycemic condition. Factors such as oxidative stress, overnutrition, and foods rich in glycat ing agents promote the formation of AGEs even in nondiabetic condition (4). Oral AGEs foster insulin resistance and diabetes by down-regulation of anti-AGE receptor-1(AGER1), sirtuin 1, and up-regulation of receptor for AGEs (RAGE) (5). AGEs affect glucose uptake, transport and pro-
target insulin resistance in adipocytes (6). While in skeletal muscle cells AGEs inhibit insulin action, mediated through RAGE (7). The AGE-RAGE axis induces oxidative stress, activates proinflammatory pathways and has been considered as a principal pathway in the pathogenesis of diabetes and its complications (8). AGE interacts with RAGE in different cells and tissues, contributing to pathogenesis in diabetes (9). By and large, AGEs contribute to development of insulin resistance leading to diabetes, as well as in the pathogenesis of diabetic complications. Therefore, analysis of plasma AGEs can possibly provide information about the severity of diabetes.

Human serum albumin (HSA), one of the most abundant plasma proteins, is highly glycated and contributes predominantly to the plasma AGEs. Apart from its role in pathogenesis, AGE-modified HSA (AGE-HSA) has been suggested as an alternative diagnostic marker to glycated hemoglobin (10). Although HbA1c is considered the “gold standard” marker, reflecting the glycemic status over the period of 8–10 weeks (1, 10), factors like anemia, blood loss, splenomegaly, and iron deficiency affect HbA1c levels (11). AGE-HSA reflects glycemic status over the preceding 3–4 weeks and has been recommended in gestational diabetes (12). In diabetes, the levels of AGE-HSA increase and were found to be positively correlated with hyperglycemia (13, 14). In addition, several recent studies have suggested that the levels of AGE-HSA are associated with prediabetic condition (15) and microalbuminuria (16). Therefore, quantification of AGE-HSA is of utmost clinical significance. Thus, understanding the site-specific modification and their dynamic transformation to heterogeneous AGEs is quite critical for mass spectrometric quantification.

AGEs can be quantified by various approaches, including colorimetric assay, ketoamine oxidase assay, enzyme-linked boronate affinity chromatography assay, fluorescence spectroscopy, boronic acid affinity chromatography assay, and mass spectrometry (MS) (17). Among these approaches, MS offers precise characterization of protein glycation, including the amino acid involved in the modification. Most of the AGEs reported in vitro and in vivo were discovered by MS-based techniques (18). AML modification has been extensively studied by different MS approaches. The fragmentation pattern and diagnostic ions for AML rearrangement product has been well established (19, 20). Further specific neutral loss ions of 162 Da, 120 Da, and 84 Da and water loss of 36 Da arising from hexose moiety of glycated peptide were also considered as signature ions to validate the glycation of peptides in HSA (21, 22). Similar characteristic patterns of water loss (18, 36, and 54 Da) ions and immonium ions derived from lysine arising from AML-modified peptide were also used to identify glycated peptides (23, 24). Diagnostic ions serve as the most reliable way of identifying glycated peptide by tandem mass spectrometry. Thus, having a good MS/MS fragment ion is key for precise characterization of glycation. However, the ratio of in vivo AGE-modified to unmodified protein is significantly low, which limits better MS/MS. Therefore, to achieve efficient identification, enrichment of glycated peptides using boronate affinity chromatography (BAC) was adopted prior to MS analysis (25). Further, by using a combination of immunodepletion, enrichment and fractionation strategies, a total of 7,749 unique glycated peptides corresponding to 1,095 native human plasma proteins, 1,592 in vitro glycated human plasma proteins, and 1,664 erythrocyte proteins were identified (26). In these lines, we have previously reported a database search approach for the identification of glycated peptide in a crude or nonenriched sample by untargeted MS/MS or data-independent workflow (27). Glycation is a chronic process; a given protein can undergo dynamic heterogeneous transformations as these proteins have varying biological lifespans, influencing the function of a protein. Thus, to assess the degree of glycation at a given pathophysiological condition, precise identification of glycation becomes critical. In this regard, a stable-isotope-dilution tandem mass spectrometry method was employed for simultaneous analysis of CML and CEL in hydrolysates of plasma proteins (28), and 13C6-glucose was utilized to quantify glycated proteins in the plasma and erythrocytes (29, 30). In a recent study, the glycation-sensitive peptides of HSA that could serve as markers for early diagnosis of type 2 diabetes were quantified by using an MS-based 18O-labeling technique (31). However, most of the previous studies have focused on AML modification, rather than other AGE modification. In fact, CML and CEL are the predominant AGEs, constituting up to 80% of total AGEs (32, 33). Diagnostic reporter ions for CML and CEL were reported recently by Prof. Ralf Hoffmann’s group (34). Here, for the first time, we report comprehensive development of an MS/MS fragment ion library for AML, CML, and CEL modifications of albumin. Further, fragment ion library was used as reference for quantification of AML-, CML-, and CEL-modified peptides of albumin in clinical plasma of healthy, prediabetic, diabetic, and microalbuminuria. Targeted SWATH analysis has led to quantification of 13 glycated peptides representing nine lysine sites. These peptides could serve as novel markers in diabetes.

**EXPERIMENTAL PROCEDURES**

Clinical Details—Subjects were recruited from Dr. Mohan’s Diabetess Specialities Centre, Chennai, India. The study was approved by the institutional ethics committee of Madras Diabetes Research Foundation, and prior written informed consent was obtained from all the study subjects. The study was performed in accordance to the Helsinki Declaration. Healthy control, prediabetic, diabetic, and microalbuminuria patients other than known history of cancer, hematuria, hypothyroidism, and history of any known infection or inflammatory diseases were included in this study. Peripheral blood was collected in EDTA vacutainers (BD Biosciences, USA). The plasma was separated by centrifugation at 1500 × g for 15 min and aliquots were stored at −80 °C. The biochemical analyses were performed immediately after the sample collection. Descriptive characters and
Targeted Quantification of Glycated Peptides of HSA in Diabetic Plasma

Fig. 1. Overview of the complete study design.

diagnostic parameters, including fasting blood glucose, HbA1c, oral glucose tolerance test, postprandial blood sugar, lipids, urea, creatinine, and microalbumin were measured (Supplemental Table 1). The fasting plasma samples were categorized into four groups, viz., from healthy control subjects (normal glucose tolerance (NGT) ), prediabetes [ impaired glucose tolerance (IGT) ], patients with type 2 diabetes mellitus (T2DM ), and diabetics with microalbuminuria [MIC] as per the World Health Organization Consulting Group Criteria. Equal volumes of two plasma samples with similar HbA1c (deviation of < 0.2%) were pooled, and three such pooled plasma in each group were used for mass spectrometric technical triplicate analysis.

Study Design—Overview of complete study design is shown in Fig. 1. First, the AGE-HSA MS/MS fragment ion library with diagnostic ions was established for glycation modifications AML, CML, and CEL. Diagnostic signature ions were validated for glycated peptides in diabetic plasma using targeted parallel reaction monitoring workflow. Further, based on the fragment ion library information, glycated peptides in healthy control, prediabetic, diabetic, and microalbuminuria plasma were quantified by using SWATH to discover candidate peptide biomarkers to assess the extent of glycation in diabetes. The results of SWATH were verified by MSE, another label-free quantification method. Two-way analysis of variance (ANOVA) was performed to assess the statistical significance of quantified glycated peptides.

Materials—All the chemicals were procured from Sigma-Aldrich (Sigma-Aldrich, MO, USA). MS-grade solvents (acetonitrile (ACN), water, and methanol) were procured from J T. Baker (J T. Baker, PA, USA). RapiGest was procured from Waters (Waters Corporation, MA, USA). Membrane filters of 3 and 30 KDa cut off were procured from Millipore (Millipore, MA, USA).

Synthesis of AGE-Modified HSA—
(a) AGE-HSA—AGE-HSA was synthesized as reported elsewhere (35, 36). Briefly, HSA (50 mg/ml) was dissolved in 10 ml of 0.2 M sodium phosphate buffer (pH 7.4) containing 0.5 M of glucose and 0.05% sodium azide, sterilized by ultrafiltration (0.22 μm filter), and incubated for 7 days at 37 °C. The samples were then extensively washed with PBS using 30 kDa cut-off filters.
(b) Chemical Synthesis of CML- and CEL-Modified HSA—CML- and CEL-modified HSA was synthesized as reported elsewhere (36, 37). Briefly, HSA (50 mg/ml) and sodium cyanoborohydride (0.15 M) were dissolved in 0.2 M sodium phosphate buffer (pH 7.4), to which either glyoxylic acid (GA) (0.045 M) or methylglyoxal (MG) (0.05 M) was added to a final volume of 10 ml for the synthesis of CML- or CEL-modified HSA, respectively. The solution was incubated for 24 h at 37 °C, followed by washing with PBS using 30 kDa cut-off filters.

Sample Preparation for MS Analysis—In-solution tryptic digestion: Equal amounts of protein (100 μg) of AGE-modified HSA and clinical plasma proteins were diluted with 100 μl of ammonium bicarbonate buffer (50 mM) containing 0.1% RapiGest, followed by incubation at 80 °C for complete proteome solubilization. The denatured proteins were then reduced with DTT (0.1 μl) at 60 °C for 15 min, followed by alkylation with iodoacetamide (0.2 μl) at room temperature in the dark for 30 min. The proteins were digested with proteomic grade trypsin at 1:50 enzyme to substrate ratio overnight at 37 °C. The digestion reaction was stopped by adding concentrated HCl and incubated for 10 min at 37 °C before being centrifuged. The peptides were desalted by using C18 Zip tip (Millipore, MA, USA) and concentration by vacuum centrifuge and stored at −20 °C until further use.

LC-MS Analysis—Instrument-specific methods and settings [(LC-HR/AM Q-Excutive Orbitrap and parallel reaction monitoring), (Triple TOF 5600 (DDA and SWATH-MS), (label-free LC-MS² on SYNAPT HDMS)] were used for the construction of the fragment ion library and quantification of glycated peptides are described below.

(a) LC-HR/AM Q-Excutive Orbitrap—
LC Separation—Peptide digests (1.5 μg) were separated by using Accela 1250 UHPLC (Thermo Fisher Scientific) equipped with a Hypersil Gold C18-reverse phase column (150×2.1 mm, 1.9 μm). The sample was loaded onto the column with 98% of mobile phase A (100% water, 0.1% formic acid (FA)) and 2% of mobile phase B (100% ACN, 0.1% FA) at 350 μl/min flow rate. Peptides were eluted with a 45 min linear gradient of 2 to 40% mobile phase B. In case of plasma samples, the LC method was extended to 120 min with a linear gradient of 2 to 50% of mobile phase B. The column temperature was set to 40 °C and auto sampler at 4 °C.

Full MS/dd-MS2 Acquisition—All samples were analyzed on hybrid quadrupole Q-Excutive Orbitrap MS. The instrument tune parameters were optimized for the better results as: spray voltage 4,200 V, capillary temperature 320 °C, heater temperature 200 °C, S-lens RF value 55, sheath and auxiliary gases pressure were 30 and 8 psi, respectively. The samples were acquired in positive ionization mode in data-dependent manner using a top-five method with scan range from 350–1,800 m/z. MS spectra were acquired at a resolution of 70,000 with maximum injection time (IT) of 120 ms and automatic gain control (AGC) value of 1 × 6 e6 ions; MS/MS spectra were acquired at 17,500 resolution with maximum IT of 120 ms and AGC value of 1 × 6 e5 ions. Precursor’s selectivity was performed at an isolation width of 3 m/z, under fill ratio of 0.3%, and dynamic exclusion time of 15 s. The peptide fragmentation was performed in high energy collision induced dissociation (HCD) cell using normalized HCD at 30 eV.

Targeted MS/dd-MS2 (Parallel Reaction Monitoring)—Peptide digests were acquired with optimized tune parameters in targeted/dd-MS2 mode by using glycated peptide ion m/z inclusion list (Supplemental Table 2). The isolation width was set to 1 m/z for the selection of precursor. MS spectra were acquired at a resolution of 70,000 with maximum IT of 120 ms and AGC value of 5 × 6 e4 ions. The peptide fragmentation was performed at a resolution of 17,500, maximum IT of 120 ms, AGC value of 2 × 6 e4 ions, and normalized HCD at 30 eV.

LC-HR/AM Q-Excutive Orbitrap mass spectrometric data were processed by using Proteome Discoverer, Version 1.4.0.288, (Thermo Fisher Scientific). The SEQUEST HT (a computer algorithm for database search) was used for peptide identification. The data were searched against HSA protein database (P02768-UniProt). The search was performed using the following parameters: Peptide and fragment mass tolerance were 10 ppm, 0.5 Da, respectively, with two missed cleavages and 1% false discovery rate. Search criteria in-

Human serum albumin
+ Glyoxylic acid
Glucose
Methylglyoxal
Heterogeneous AGEs

In-solution trypsin digestion

LC-HRAM-Quadrupole-Orbitrap MS/MS

Establishment of diagnostic fragment ions of AML, CML and CEL
[Validation by targeted proteomics PRM]

Targeted and global LC-MS/MS workflows SWATH and MS² for glycated peptide quantification

Validation with diabetic clinical samples
included fixed and variable modifications as carbamidomethylation (C) and oxidation (M), respectively. Additional variable lysine-specific glycation modifications AML (162.02), CML (162.02), and CEL (566.6 (1,697.9 Da) were considered.

(b) Analyses on Triple TOF 5600 (DDA and SWATH-MS)—

LC Separation—Peptide digests (3 μg) were separated by using an Eksigent MicroLC 200 system (Eksigent, Dublin, CA) equipped with Eksigent C18-reverse phase column (100*0.3 mm, 3 μm, 120 Å). The sample was loaded onto the column with 97% of mobile phase A (100% water, 0.1% FA) and 3% of mobile phase B (100% ACN with 0.1% FA) at 8 μl/min flow rate. Peptides were eluted with a 120 min linear gradient of 3 to 50% mobile phase B. The column temperature was set to 40 °C and auto sampler at 4 °C. The same chromatographic conditions were used for both DDA and SWATH acquisition.

Full MS/dd-MS2 Acquisition (DDA for Creating SWATH Library)—
All samples were analyzed on AB-SCIEX 5600 Triple TOF mass spectrometer in positive and high-sensitivity mode. The dual source parameters were optimized for better results: ion source gases GS1, GS2, curtain gas at 25 psi, temperature 200 °C, and ion spray voltage floating at 5,500 V. The accumulation time in full scan was 250 ms for a mass range of 350–1,800 m/z. The parent ions are selected based on the following criteria: ions in the MS scan with intensity more than 120 counts per second, charge stage between +2 to −5, and mass tolerance 50 mDa. Ions were fragmented in the collision cell using rolling collision energy (CE) with an additional CE spread of ± 15 eV.

Peptide Spectral Library and Database Search—Glycated peptides derived from synthetically glycated HSA were acquired in technical triplicate using the above-mentioned DDA method. DDA mass spectrometric files were searched using ProteinPilot software, Version 4.0.8085 (AB SCIEX, MA, USA) with the Paragon algorithm against human serum albumin protein database (P02768-UniProt) at 1% false discovery rate. The ProteinPilot output file (.group) was used as a standard peptide spectral library.

SWATH MS—In SWATH-MS mode, the instrument was specifically tuned to optimize the quadrupole settings for the selection of precursor ion window of 25 m/z wide. Using an isolation width of 26 m/z (containing 1 m/z for the window overlap), a set of 34 overlapping windows was constructed covering the precursor mass range of 400–1,250 m/z. SWATH MS/MS spectra were collected from 100 to 2,000 m/z. Ions were fragmented in the collision cell using rolling collision energy with an additional CE spread of ± 15 eV. An accumulation time (dwell time) of 96 ms was used for all fragment-ion scans in high-sensitivity mode, and for each SWATH-MS cycle a survey scan in high-resolution mode was acquired for 100 ms, resulting in a duty cycle of 3.33 s. The source parameters were similar to that of DDA acquisition.

SWATH analysis was performed for three biological replicates and technical triplicates each from healthy control, prediabetic diabetic, and microalbuminuria. Each biological replicate was a pool of two plasma samples with similar HbA1c. The spectral alignment and targeted data extraction of SWATH-MS data were performed using Peakview software, Version 1.2.03 (AB SCIEX, MA, USA). The peptide data (.MRKVW) files were used for quantification of glycated peptides of HSA using Markerview software, Version 1.2.1.1 (AB SCIEX, MA, USA). Normalization was performed using total area sum. The peptides with a p value ≥ 0.05 were considered for quantification.

(C) Analyses on SYNAPT HDMs—Protein digest (400 ng) was analyzed by LC-MS6 workflow using nanoACQUITY (UPLC) online coupled to Synapt HDMS system (Waters Corporation, MA, USA) equipped with a nanolockspray ion source with flow rate of 300 nL/min (external lock mass standard: glu-fibrinopeptide (m/z 785.8426)). Sampling of the lock spray channel was performed every 30 s, and the system was tuned for to a resolution of about 9000 in positive V-mode, and calibrated using glu-fibrinopeptide infusion. Peptide samples were injected online onto a 5 μm Symmetry C18 trapping column (180 μm x 2 cm) at a flow rate of 5 μL/min. Peptides were separated by in-line gradient elution onto bridged ethyl hybrid (1.7 μm x 75 μm x 250 mm) column at a flow rate of 300 nL/min using a linear gradient from 2 to 40% B over 90 min (A. 100% water with 0.1% FA, B. 100% ACN with 0.1% FA). Acquisition was performed in positive V mode in a mass range of 50–2,000 m/z with a scan time of 0.7 s with alternating low (4 eV) and high (15 to 40 eV) collision energy. A capillary voltage of 3 kV, source temperature of 80 °C, and cone voltage of 32 V were maintained during the analyses. LC-MSE data were processed using Protein Lynx Global Server (PLGS, Version 2.5.1) for identification and quantification of glycated peptides of albumin (27, 35).

Structure Analysis—Three-dimensional structure of albumin highlighting lysine residues image was generated with the help of using a PyMOL Molecular Graphics System (Schroedinger, LLC, New York). Electrostatic surface potentials were calculated using Blues with default values as implemented in the Blues web server (36).

Statistical Analysis—Significant differences between glycated peptide m/z within diabetes condition and across m/z were determined by using two-way ANOVA followed by Tukey’s multiple comparison test. NS represents a nonsignificant difference within the diabetic conditions and/or across m/z.

RESULTS

HSA, one of the most abundant plasma protein, is highly glycated and contributes predominantly to the plasma AGEs. Certain lysine residues of HSA are more prone to undergo glycation modification, which are also called glycation/glucose-sensitive sites (31). As these sites are continuously exposed to higher glucose concentration, these sites can possibly undergo sequential AGE modifications followed by initial Amadori rearrangement. CML and CEL constitutes about 80% of total AGEs. Therefore, in this study, a diagnostic ion library for AML-, CML-, and CEL-modified peptides of HSA was established and quantified by targeted SWATH workflow in clinical plasma to evaluate their use as diagnostic peptides.

Establishment of Diagnostic Fragment Ion Library for AML, CML, and CEL Peptides of HSA—The fragment ion library was constructed sequentially in four steps as depicted in Supplemental Fig. 1: (i) the HSA was modified with glucose or GA or MG to obtain AML-, CML-, and CEL-modified peptides, respectively (Fig. 2); (ii) the AGE-modified HSA was subjected to tryptic digestion, and the peptides formed were analyzed by using high resolution accurate mass spectrometer (Q Exactive Hybrid Quadrupole Orbitrap MS); (iii) peptide identification and manual validation for consensus MS and MS/MS spectra; and (iv) construction of final diagnostic fragment ion library for AML, CML, and CEL peptides of albumin (Table I and Supplemental Table 3).

The search-algorithm-identified glycated peptides were manually validated by inspecting each MS/MS spectrum as described earlier by (27). Briefly, at MS, each AGE-modified peptide precursor was reviewed for presence of unmodified peptide precursor. For example, CML-modified precursor ion m/z 566.6 (1,697.9 Da MH+) corresponding to peptide (KTVQVSTPLLVEVSR) was inspected for the presence of its.
unmodified precursor ion m/z 547.31 (1,639.9 Da). Such manual interrogation was carried out for the entire glycated peptide precursor ions having increased mass of 162.053, 58.005, and 72.021 Da corresponding to AML, CML, and CEL modifications, respectively. At MS/MS, glycated precursor fragment ions were carefully evaluated for glycated lysine ions of m/z 291.14 (128.09 + 162.051), m/z 187.09 (128.09 + 58.005), and m/z 201.11 (128.09 + 72.021) corresponding to AML, CML, and CEL, respectively, followed by increment shift in mass across b or y ion series, depending on the site of modification in peptide. An example of modification at N-terminal lysine, CML-modified K*VPQVSTPTLVEVSR (m/z - 566.652) peptide spawned signature diagnostic b series fragment ions b_1^- = 187.10, b_2^- = 286.17, b_3^- = 383.22, b_4^- = 511.28, b_5^- = 610.35, b_6^- = 697.38, b_7^- = 798.43, and b_8^- = 996.53 bearing 58 Da mass shift across b ion series (Fig. 3). An example of modification at C-terminal, CML modified RHPFYAPELLFFAK*R (m/z - 705.037) peptide spawned signature diagnostic y series fragment ions y_2^- = 894.44, b_6^- = 965.48, b_9^2^- = 576.28, b_10^2^- = 1,222.60, b_11^- = 1,321.66, b_12^- = 1,392.70, y_7^- = 859.47, y_8^- = 1,006.54, y_9^- = 1,077.58, and y_10^2^- = 617.34 (Supplemental Fig. 3). In some instances, there were poor MS/MS resulting in identification of false positives. In order to rule out any such identification, the presence of three consecutive fragment ions bearing modification was adopted as a necessary criterion. All such glucose-, GA-, and MG-induced glycated peptides are listed in Table I (corresponding fragment ions and MS/MS spectra are provided in supplemental file 1A and supplemental file 4 (4A, B, and C), respectively). In agreement with the rule of consecutive ions, some of the peptides were consented as true modifications for their better MS/MS fragmentation pattern, where either y or b ion were bearing the shift of modified mass, which are listed in Supplemental Table 3 (corresponding peptide fragment ions and MS/MS spectra are provided in supplemental file 1B and supplemental file 5, respectively). A list of all the corresponding unglycated peptides are listed in Supplemental Table 6.

Together, 50 glycated peptides were identified for AML-, CML-, and CEL-modified HSA. Each of the glycated protein showed 20, 17, and 13 modified peptides induced by glucose GA and MG, respectively. These 50 modified peptides repre-
| SLN | Targeted site | Modified site | Peptide start-end | AGE-modified peptide sequence | Peptide MH+ Da | Monoisotopic m/z Da (mmu/ppm) | CS | RT | XC | MC | Glycation Diagnostic fragment ions |
|-----|---------------|---------------|-------------------|-------------------------------|---------------|--------------------------------|-----|-----|----|----|-----------------------------------|
| 1   | 160           | 159–160       | LVPR/EVDVMTAFDHN   | 936.96 (+1.11/+1.11)           | 927.49        | 1055.58 (-0.69/-1.54)         | 1   | 2   |    |    | CML 105.58 (0.00)                  |
| 2   | 160 & 161     | 159–160       | LVPR/EVDVMTAFDHN   | 924.11 (-0.69/-1.54)           | 927.49        | 1055.58 (-0.69/-1.54)         | 1   | 2   |    |    | CML 105.58 (0.00)                  |
| 3   | 402           | 397–403       | VDFER/KLPVEQPQKQNC/ | 937.48 (+1.12/+1.13)           | 638.28        | 921.43 (-0.70/-1.83)          | 1   | 2   |    |    | CML 921.43 (0.00)                  |
| 4   | 183           | 169–168       | RHPF/VAYELLFKAKR   | 930.45 (+1.12/+1.13)           | 157.11        | 769.37 (-0.03/-1.70)          | 1   | 2   |    |    | CML 769.37 (0.00)                  |
| 5   | 437           | 435–438       | YTK*VQVSTPVLVEK    | 959.38 (+1.22/+1.27)           | 265.11        | 226.11 (-0.03/-1.70)          | 1   | 2   |    |    | CML 226.11 (0.00)                  |
| 6   | 437 & 438     | 435–438       | YTK*VQVSTPVLVEK    | 959.38 (+1.22/+1.27)           | 265.11        | 226.11 (-0.03/-1.70)          | 1   | 2   |    |    | CML 226.11 (0.00)                  |
| 7   | 438           | 436–438       | K*VPQVSTPVLVEKSRNLK | 562.30 (+0.45/+0.44)           | 187.10        | 286.17 (-0.03/-1.70)          | 1   | 2   |    |    | CML 286.17 (0.00)                  |
| 8   | 438           | 436–438       | K*VPQVSTPVLVEKSRNLK | 562.30 (+0.45/+0.44)           | 187.10        | 286.17 (-0.03/-1.70)          | 1   | 2   |    |    | CML 286.17 (0.00)                  |
| 9   | 183           | 170–184       | HPPF/VAYELLFKAKR   | 956.20 (+0.45/+0.44)           | 187.10        | 286.17 (-0.03/-1.70)          | 1   | 2   |    |    | CML 286.17 (0.00)                  |
| 10  | 236           | 230–230       | FGIER/KAVAVAR      | 738.91 (-0.62/-1.94)           | 894.44        | 965.48 (-0.62/-1.94)          | 1   | 2   |    |    | CML 965.48 (0.00)                  |
| 11  | 426           | 426–426       | QC/ELFGQYKQGDNALLVR | 886.43 (-0.60/-1.63)           | 990.56        | 914.66 (-0.60/-1.63)          | 1   | 2   |    |    | CML 914.66 (0.00)                  |
| 12  | 549           | 549–558       | K*GTIALVEK        | 590.35 (+0.34/+0.47)           | 187.10        | 315.16 (-0.03/-1.70)          | 1   | 2   |    |    | CML 315.16 (0.00)                  |
| 13  | 229           | 229–230       | C*ASLOGKYSER      | 461.53 (-0.20/-0.48)           | 508.25        | 694.35 (-0.20/-0.48)          | 1   | 2   |    |    | CML 694.35 (0.00)                  |
| 14  | 547           | 549–557       | RPP/CSALEDEVTYKPERAETFTADCC/ | 922.62 (+3.94/+4.37)           | 561.24        | 702.35 (-0.43/-1.37)          | 1   | 2   |    |    | CML 702.35 (0.00)                  |
| 15  | 183           | 169–184       | RHPF/VAYELLFKAKR   | 930.45 (+1.12/+1.13)           | 157.11        | 769.37 (-0.03/-1.70)          | 1   | 2   |    |    | CML 769.37 (0.00)                  |
| 16  | 490           | 496–498       | RMPC/AEDYLSVNLQGC/ | 884.39 (+0.60/+0.80)           | 187.10        | 315.16 (-0.03/-1.70)          | 1   | 2   |    |    | CML 315.16 (0.00)                  |
| 17  | 426           | 424–424       | QC/ELFGQYKQGDNALLVR | 886.43 (-0.60/-1.63)           | 990.56        | 914.66 (-0.60/-1.63)          | 1   | 2   |    |    | CML 914.66 (0.00)                  |
| 18  | 958           | 958–960       | K*VAASQAGGL        | 607.36 (-0.18/-0.30)           | 213.12        | 213.12 (-0.18/-0.30)          | 1   | 2   |    |    | CML 213.12 (0.00)                  |
| 19  | 10            | 40–44         | FKAGOOCNP       | 433.54 (-0.56/-1.29)           | 148.07        | 349.18 (-0.56/-1.29)          | 1   | 2   |    |    | CML 349.18 (0.00)                  |

**Glycation** induced modifications:
- *carbamidomethyl (57.02146 Da), CS—charge state, RT—retention time, XC—Xcorr, MC—missed cleavage.

**Methylation** induced modifications:
- Static modification: *carbamidomethyl (57.02146 Da), CS—charge state, RT—retention time, XC—Xcorr, MC—missed cleavage.
presented 23 lysine modified sites. K549, K438, and K183 sites were modified six times, followed by K375 and K490 (each four times), suggesting that these sites were more sensitive to glycation modification than others (Table I and Supplemental Table 3). MS and corresponding XICs of all 50 glycated peptides along with unmodified control peptides are provided in supplemental files 3A, 3B, and 3C.

The reliability of the diagnostic fragment ion library for AML-, CML-, and CEL-modified peptides of HSA was validated by parallel reaction monitoring of selected glycated peptide m/z (inclusion list is provided as Supplemental Table 2). The resultant ion chromatograms were extracted with a mass tolerance of 5 ppm, and the presence of at least two corresponding diagnostic fragment ions were confirmed manually (Supplemental Table 2, and corresponding XICs are provided in supplemental file 3).

Quantification of HSA Glycated Peptides in Clinical Plasma—AGE-modified HSA peptides from pooled plasma samples of healthy control, prediabetic, diabetic, and microalbuminuria were quantified by using label-free targeted SWATH workflow. In SWATH analysis, the intensity fold change of 13 glycated peptides listed in Fig. 4 were found to be consistently higher in prediabetes, diabetes, and microalbuminuria in comparison with the control (Supplemental Table 4).

Further, two-way ANOVA was performed to assess the statistical significance of intensity fold change of glycated HSA peptides among prediabetes, diabetes, and microalbuminuria. Concentrations of the quantified m/z and ANOVA results are listed in Supplemental Table 4 and Supplemental Table 5, respectively. Two way ANOVA results showed statistically significant interaction with a total variance of 29.63% at \( p = .0001 \) between glycated peptides m/z and different diabetic conditions (prediabetic, diabetic, and microalbuminuria). Tukey’s multiple comparisons test suggested that, out of 13 consistent glycated peptides, fold expression of four m/z viz. 600.8, 808.6, 665.5, and 886.4 was significant when compared across three diabetic conditions (i) m/z - 600.8, Site - K549, sequence - K^{CEL*}QTALVELVK was found to be significant in diabetes (\( p = .01 \)) and microalbuminuria (\( p = .02 \)) as compared with prediabetes; (ii) m/z - 808.6, Site - K490, sequence - MPC*AEDYLSVVLNQKL*VHEK^{CML*}TPVSDR was found to be significant in diabetes (\( p = .01 \)) and microalbuminuria (\( p = .02 \)) as compared with prediabetes; (iii) m/z - 665.5, Site - K88, sequence - TC*VADESAENC*DK^{AML*}SLHTLFGDK was found to be more significant in diabetes (\( p = .0005 \)) as compared with microalbuminuria and significant in prediabetes (\( p = .03 \)) as compared with microalbuminuria; and (iv) m/z - 886.4, Site - K426, sequence - QNC*ELFEQLGEYK^{CML*}.
FQNALLVR was found to be more significant in diabetes \((p = .0007)\) and prediabetes \((p = .003)\) as compared with microalbuminuria (Fig. 4). Further, to determine the significance of intensity, the fold change of each glycated peptide was compared across all glycated peptides (Supplemental Fig. 4). Glycated peptide ion \(m/z\) 886.4 and \(m/z\) 433.5 were found to be significant in prediabetes, while \(m/z\) 886.4, \(m/z\) 433.5, and \(m/z\) 665.5 were found to be significant in diabetes and \(m/z\) 601.3 and \(m/z\) 593.8 were found to be significant in microalbuminuria. Further, the results of SWATH analysis were verified by MS² workflow. Out of 13 glycated peptides, nine peptides \((m/z\) 645.8, 593.8, 601.3, 528.3, 834.6, 808.6, 665.5, 529.0, and 886.4) intensity fold change was found to be higher in prediabetes, diabetes, and microalbuminuria in comparison with control and showed similar trend with the SWATH results (Supplemental Fig. 5 and Supplemental Fig. 6), while four other peptides \((m/z\) 600.8, 639.8, 433.5, and 951.4) showed increased intensity fold change in diabetic condition by only SWATH analysis.

**Glycated HSA Model—In vitro** glucose-, GA-, and MG-induced glycated HSA representing glycation sites are shown in three-dimensional structure of HSA (Supplemental Fig. 7). From the surface electrostatic distribution calculation, it was clear that most of the modified lysine residues were situated near the positive or neutral groove. CML modifications by GA were predominantly found in higher electropositive grooves, suggesting CML modification requires highly positive local surface environment (Supplemental Fig. 8).

**DISCUSSION**

Historically, mass spectrometry has been used for precise characterization of protein glycation and has been reviewed in great detail (17). Quantitative capabilities of mass spectrometry have been recently extended to quantify glycated plasma proteome, including albumin (29–31). The majority of the previous studies dealt mainly with Amadori-modified peptides of albumin. However, it is important to quantify CML- and CEL-modified albumin, as these modifications constitute the predominant AGEs. Several mass-spectrometry-based targeted quantification approaches, including multiple reaction monitoring, parallel reaction monitoring, and SWATH, have become powerful tools and rely heavily on fragment ion library (39, 40). In this context, we constructed a fragment ion library for the synthetically AML-, CML-, and CEL-modified peptides of albumin by using high-resolution accurate mass spectrometer followed by rigorous inspection and validation of MS/MS spectra. Furthermore, using the ion library, AML-, CML-, and CEL-modified albumin peptides were quantified by targeted SWATH analysis in the clinical plasma. This study has led to the identification, characterization, and quantification of 13 glycated peptides in the clinical diabetic plasma comprising 4-AML, 7-CML, and 2-CEL modification representing nine lysine sites of albumin (K36, K88, K160, K161, K183, K375, K438, K490, and K549). Among these sites, K549, K438, K490, and K549 were highly sensitive for glycation modification as they had both AML and CML modifications, and K549 had additional CEL modification, which was identified (31). Although it is intriguing that only one lysine site \(i.e\). K549 was found to be CEL modified, this result strongly supports a recent study where no CEL-modified peptide was identified (34).

HSA is a major cargo protein of fatty acids, small proteins, metabolites, drugs, etc. HSA contains a total of 58 surface-exposed lysine residues rendering their accessibility for interaction with various blood constituents including glucose. Only a few lysines are surface accessible to undergo glycation modification, resulting in impaired antioxidant and cargo capabilities of HSA (41). Similar to HSA modification by malondialdehyde (42), a highly positive local environment expedites glycation reaction, determining the site specificity of glycation. Recent studies that report the structural and biological impacts of albumin glycation also clearly indicate the plausi-
bility of glycated albumin to be used as a specific marker for diabetes (43).

In conclusion, this study reports the development of fragment ion library for AML-, CML-, and CEL-modified peptides of albumin. This library will be very useful for quantification of AGE-modified peptides of albumin by targeted mass spectrometric approaches. Using this fragment ion library, novel AGE-modified peptides were quantified. Precursor ions appertaining to K549, K438, K490, K88, and K375 could serve as potential novel markers for assessing the degree of glycation or diabetic glycemic status in general and m/z 645.8 (K^AML*QTALVELVK), m/z 593.8 (K^CML*QTALVELVK), m/z 600.8 (K^CEL*QTALVELVK), m/z 601.3 (K^AML*VPQVSTPTVEVSVR), m/z 528.3 (K^CML*VPQVSPTPTVEVSVR), m/z 834.6 (MPC^AEDLYSVLNOCL*VLEHK^AML*TPVS), m/z 808.6 (MPC^AEDLYSVLNOCL*VLEHK^CML*TPVS), m/z 665.5 (TC^VADESAENC*D^K^ML*SLHTLFGDK), m/z 639.5 (TC^VADESAENC*D^CML*SLHTLF-GDK), and m/z 886.4 (QNC^ELFEQLGKEY^CML*FGNALLVR) in particular. However, as this is a pilot study involving a limited number of samples, it is important to analyze a larger patient cohort to consider these peptides as biomarkers.

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