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Discovery of Free Glycated Amines and Glycated Urea in Diabetic Plasma: Potential Implications in Diabetes

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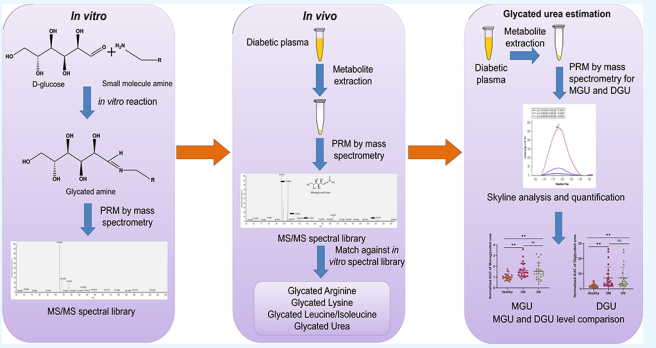
ABSTRACT: The role of protein glycation in the pathogenesis of diabetes has been well established. Akin to proteins, free amino acids and other small-molecule amines are also susceptible to glycation in hyperglycemic conditions and may have a role in the pathogenesis of the disease. However, information about glycation of free amino acids and other small-molecule amines is relatively obscure. In the quest to discover small-molecule glycated amines in the plasma, we have synthesized glycated amino acids, glycated creatine, and glycated urea, and by using a high-resolution accurate mass spectrometer, a mass spectral library was developed comprising the precursor and predominant fragment masses of glycated amines. Using this information, we report the discovery of the glycation of free lysine, arginine, and leucine/isoleucine from the plasma of diabetic patients. This has great physiological significance as glycation of these amino acids may create their deficiency and affect vital physiological processes such as protein synthesis, cell signaling, and insulin secretion. Also, these glycated amino acids could serve as potential markers of diabetes and its complications. While other amines, such as creatinine and urea, accumulate in the plasma and act as biomarkers of diabetic nephropathy. For the first time, we report the detection of glycated urea in diabetic plasma, which is confirmed by matching the precursor and fragment masses with the *in vitro* synthesized glycated urea by using $^{12}C_6$ and $^{13}C_6$-glucose. Further, we quantified glycated urea detected in two forms, monoglycated urea (MGU) and diglycated urea (DGU), by a targeted mass spectrometric approach in the plasma of healthy, diabetic, and diabetic nephropathy subjects. Both MGU and DGU showed a positive correlation with clinical parameters, such as blood glucose and HbA1c. Given that urea gets converted to glycated urea in hyperglycemic conditions, it is crucial to quantify MGU and DGU along with the urea for the diagnosis of diabetic nephropathy and study their physiological role in diabetes.



INTRODUCTION

Protein glycation, a nonenzymatic reaction between reducing sugars and proteins, has been a well-studied process in diabetes.[1] This reaction was first reported by Louis Camille Maillard in 1912 while heating amino acids and reducing sugars. Thus, it is also referred to as Maillard's reaction. Since then, glycation has been studied; however, it is mainly limited to proteins.[2] Especially, the glycation of abundant large molecular weight proteins such as hemoglobin and albumin is documented in great detail by various researchers.[3] Glycated hemoglobin (HbA1c) is used as a diagnostic marker for diabetes management.[4] Glycated albumin, on the other hand, acts as a predominant advanced glycation end product (AGE) in circulation and is known to have harmful physiological consequences through interaction with the receptor for AGE (RAGE).[5] Both of these proteins are abundant and long-lived; thus, they are more prone to undergo glycation. In contrast, the low-abundant proteins with a shorter lifespan are less likely to get glycated, although a few low-abundant proteins, such as insulin, hexokinase, catalase, superoxide dismutase (SOD), and glutathione peroxidase, are found to be glycated.[6] The impact of glycation on such low-abundance protein cannot be undermined. For example, *in vitro*, glycated insulin has a lesser binding affinity for insulin receptors and interacts with RAGE to activate oxidative stress and proinflammatory pathways.[7] Similarly, free amino acids are also likely to undergo glycation. In yeast, the formation of glycated products such as pyrraline, formyline, and maltosine from free amino acids was observed during the brewing of beer.[8] In diabetes, there is a possibility of

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Table 1. HRMS Analysis of *In Vitro* Synthesized Glycated Amines with Most Intense Fragment Ions

S. no.	glycated amines	monoisotopic mass (m/z)	observed mass (m/z)	RT (min)	fragment ions	intensity
1	monoglycated arginine	337.1723	337.1707	1.53	70.0654 112.0869 114.1026 173.1393 175.1185 217.1287	1.53e6
2	diglycated arginine	499.2251	499.2133	1.76	70.0654 114.1025 214.9622	5.70e3
3	glycated cysteine	284.0804	284.0800	1.89	100.0216 122.0269 146.0267	3.93e6
4	glycated glutamine	309.1298	309.1277	1.84	97.0285 130.0497 208.0599	3.71e5
5	glycated glutamic acid[a]	310.1138	310.1120	1.83	97.0286 148.0603 226.0708	7.25e5
6	glycated isoleucine	294.1552	294.1520	1.86	86.0966 132.1017 230.1382 258.1330	3.69e6
7	monoglycated lysine	309.1661	309.1648	1.60	84.0810 128.0704 225.1227	4.50e6
8	diglycated lysine	471.2189	471.2174	1.58	84.0810 212.1276 246.1330	1.29e5
9	glycated proline	278.1240	278.1219	1.84	128.0705 242.1017 260.1122	2.29e5
10	glycated serine	268.1032	268.1029	1.82	97.0286 130.0497 232.0811	4.88e5
11	glycated threonine	282.1189	282.1171	1.81	97.0285 144.0653 246.0965	9.03e5
12	glycated valine	280.1396	280.1389	1.80	216.1225 244.1173 262.1278	3.26e6
13	glycated alanine[a]	252.1083	252.1071	1.82	102.0552 216.0866 234.0971	4.90e5
14	glycated asparagine	295.1141	294.0034	1.83	87.0555 194.0444 211.0709	3.28e5
15	glycated aspartic acid	296.0981	296.5928	2.05	85.0286 173.9607 260.0764	1.37e4
16	glycated glycine	238.0927	239.1057	1.80	88.0395 97.0286 202.0706	1.06e5
17	glycated histidine	317.1223	318.1276	1.58	95.0605 190.0970 238.1180	2.74e6
18	glycated leucine	294.1552	294.0018	1.77	86.0967 132.1018 230.1383 258.1331	3.05e5
19	glycated methionine[a]	312.1117	312.1121	1.84	88.0396 133.0317	3.87e6

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Table 1. continued

S. no.	glycated amines	monoisotopic mass (m/z)	observed mass (m/z)	RT (min)	fragment ions	intensity
20	glycated phenylalanine	328.1396	328.8529	1.89	276.0897 186.9115 204.9221	1.13e5
21	glycated tryptophan	367.1505	367.1717	1.87	288.8596 164.9297 186.9115	6.50e3
22	glycated tyrosine	344.1345	345.1556	1.87	254.8987 165.0541 281.8988	8.11e3
23	glycated creatine	294.1301	294.1369	1.61	308.1132 90.9771 158.9640	5.28e5
24	monoglycated urea	223.0930	223.0938	1.71	206.9552 104.9925 116.9925	5.79e6
25	diglycated urea	385.1458	385.1205	1.80	135.0029 203.0526	6.09e5

[a]Glycated amines synthesized on 7th day.

free amino acids and other amines undergoing glycation under hyperglycemic conditions. However, there are no studies in the literature with respect to the glycation of free amino acids and small-molecule amines. This could be due to a lower concentration, shorter half-life, and lack of methods or libraries to identify glycated amines. In light of this, we made an attempt to discover novel small-molecule glycated amines from diabetic plasma. To this end, we have synthesized glycated forms of amino acids, urea, and creatine and, using the information on precursor and fragment masses, discovered novel glycated amines and quantified glycated urea in the plasma of healthy (H), diabetes (DM), and diabetic nephropathy (DN) subjects by using a targeted metabolomics approach.

MATERIALS AND METHODS

Materials. Reagents, such as LC-MS-grade water, methanol (MeOH), and acetonitrile (ACN), were purchased from JT Baker (PA, USA). Formic acid, sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), all 20 amino acid standards, urea, creatine, and glucose were obtained from Sigma-Aldrich (St. Louis, MO, USA). Thermo Hypersil Gold C18 column (length 150 mm, ID 2.1 mm, particle size 1.9 μM and pore size 175 Å) was procured from ThermoFisher Scientific (Lithuania, Europe).

Study Design. This study was carried out to discover novel small-molecule glycated amines in the plasma. Glycated amino acids, glycated creatine, and glycated urea were synthesized *in vitro* to identify glycated amines. The mass spectral library comprising precursor and product masses was developed for synthesized *in vitro* glycated amines. Using this information, glycated amines from the plasma of diabetic patients were identified. The plasma samples used in this study were collected in a previous study to discover risk prediction markers for diabetic nephropathy from Chellaram Diabetes Institute, Pune, India, with the approval of the Institutional Ethics Committee. A signed written informed consent was obtained from all subjects before the blood collection. The procedure for plasma collection was detailed earlier.[9]

***In Vitro* Synthesis of Glycated Amino Acids, Urea, and Creatine.** The glycation reaction was carried out by incubating 500 μL of each amino acid (100 mM), viz., alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, lysine, methionine, proline, serine, threonine, and valine with 500 μL of D-glucose (500 mM) in 50 mM phosphate buffer (pH 7.4) at 37 °C for 72 h. The concentration was 50 mM for leucine, while for tyrosine, tryptophan, and phenylalanine, it was 3.125 mM, and proportionately, glucose concentration was diluted. Similarly, glycated creatine and glycated urea were synthesized by incubating 500 μL of creatine (25 mM) and urea (100 mM) with 500 μL of glucose (500 mM). Further, the *in vitro* synthesized glycated amino acids, glycated creatine, and glycated urea were analyzed by parallel reaction monitoring (PRM) using LC-HRMS (Thermo Q-Exactive Orbitrap mass spectrometer) studies followed by XIC-based confirmation.

Extraction of Metabolites from Plasma Samples. For the extraction of metabolites, 100 μL of plasma was mixed with 400 μL of chilled methanol and incubated for 1 h at −20 °C. The precipitated protein was separated by centrifugation for 20 min at 13,000*g*, and the supernatant containing metabolites was dried by using a vacuum concentrator at 4 °C. The sample was reconstituted in 100 μL of 50% methanol, sonicated for 2 min, centrifuged for 15 min, and used for mass spectrometric analysis.[10] The extracted metabolites were analyzed to detect glycated amines by PRM as described above for *in vitro* glycated amines.

LC-HRMS Analyses. The synthesized glycated amines or plasma extracted glycated amines were acquired in PRM by using a UHPLC−high-resolution mass spectrometer (LC-HRMS, Orbitrap, ThermoFisher Scientific) equipped with a heated electrospray ionization (HESI) source. Individual glycated amine (1 μL) was loaded onto the C18 column (length 150 mm, ID 2.1 mm, particle size 1.9 μM, and pore size 175 Å). Glycated amines were eluted from the column in an isocratic mode through the mobile phase consisting of 1:1 acetonitrile and water, supplemented with 0.1% formic acid at a flow rate of 200 μL/min with 5 min runtime. The precursor masses to be detected were specified in the inclusion list of the PRM method. The mass spectrometer conditions were described earlier.[11] The precursor masses to be detected were specified in the inclusion list of the PRM method. The

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precursor ions were fragmented at a normalized collision energy of 30 eV, and the fragments were acquired at a resolution of 17,500. The AGC target was set to 2e5, the maximum IT to 100 ms, and the isolation window to 4.0 m/z. Sheath gas and auxiliary gas flow rates were set to 40 and 15, respectively. All the mass spectrometry acquisition raw files are stored on the Metabolights server (MTBLS9851).[12]

Screening and Identification of Glycated Amines from the Plasma. A list of the accurate masses of glycated amino acids, glycated creatine, and glycated urea is tabulated (Table 1). The presence of these masses was investigated by an extracted ion chromatogram. The glycated amines were confirmed by matching the precursor and fragment ions with the *in vitro* synthesized glycated amines (Table 2).

Table 2. HRMS Analysis of Glycated Amines Detected in the Diabetic Plasma

S. no.	glycated amines	monoisotopic mass (m/z)	observed mass (m/z)	RT (min)	fragment ions matching with *in vitro* synthesized
1	glycated arginine	337.1723	337.1719	1.61	60.0561
					70.0656
					114.0915
					173.1397
					217.1292
					257.1604
2	glycated lysine	309.1661	309.1656	1.67	84.0810
					128.0705
					225.1230
3	glycated leucine/ isoleucine	294.1552	294.2134	2.06	86.0967
					132.1018
					230.1384
					258.1332
4	monoglycated urea	223.0930	223.0937	1.80	104.9924
					116.9923
					135.0027
5	diglycated urea	385.1458	385.1518	1.90	203.0523

Quantification of Glycated Urea by PRM. Targeted quantification of glycated urea was performed by PRM[1]. The mass spectrometric raw data were processed and analyzed using Skyline (version 21.2.0.568) with the help of the transitions of metabolites of interest.

$^{13}C_6$-Glycated urea (2 μL) (0.4 mM) was used as the internal standard for normalization. Relative quantification of glycated urea was performed by the following equation.

$$\text{relative peak area} = \frac{(\text{peak area of glycated urea from plasma})}{(\text{peak area of } ^{13}C_6\text{-glycated urea})}$$

$^{12}C_6$-Glycated urea and $^{13}C_6$-glycated urea were synthesized by incubating 500 μL of 100 mM urea with 500 μL of 500 mM of $^{12}C_6$-glucose and $^{13}C_6$-glucose in 50 mM phosphate buffer (pH 7.4) for 72 h at 37 °C, respectively. A tandem mass spectrometry (MS/MS) was performed for both $^{13}C_6$- and $^{12}C_6$-glycated ureas to characterize the fragmentation pattern.

Correlation of Glycated Urea with Various Clinical Parameters. Correlation analysis of plasma glycated urea was carried out with all the recorded clinical parameters like fasting and postprandial blood glucose levels, HbA1c, microalbuminuria, and serum creatinine using Pearson's correlation method. Here, the data of different subject groups (H, DM, and DN) were analyzed using GraphPad Prism 8.0.2 software. In the present study, p-values less than 0.05 were considered statistically significant.[9]

RESULTS

***In Vitro* Synthesis of Glycated Amino Acids.** The *in vitro* reaction between glucose and various amino acids for 3 days led to the synthesis of 17 glycated amino acids that are listed in Table 1. Tandem mass spectrometric analysis of these glycated amino acids provided information about their fragments, which will be useful for identifying and quantifying glycated amino acids from complex matrices like plasma. Detailed information on the precursor and fragments of glycated amino acids is provided in Table 1. Among these glycated amino acids, lysine, cysteine, isoleucine, and valine showed higher intensity (Table 1, Figure S1), suggesting these are more prone to undergo modification by glucose. Alternatively, these glycated amino acids may have a higher ionization efficiency. Also, glutamic acid, alanine, and methionine were found to be glycated on the seventh day (Table 1), suggesting that these amino acids are relatively less susceptible to glycation.

In addition to amino acids, other small-molecule amines, such as urea and creatine were also found to be glycated *in vitro* (Table 1). Urea was detected in both monoglycated and diglycated forms (Table 1). Creatine, a precursor of creatinine, and urea were considered for the *in vitro* glycation reaction, as these are markers of kidney dysfunction and diabetic nephropathy.[6,10]

Identification of Glycated Amines in the Plasma of Diabetic Patients. The presence of glycated amines in plasma was confirmed by matching their precursor and fragment ions with the *in vitro* synthesized glycated amines. By this approach, the presence of glycated forms of lysine, arginine, leucine/isoleucine was detected in the diabetic plasma. Glycated lysine (m/z = 309.1661) from the plasma showed the following matching fragments (m/z = 84.0810, 128.0705, 225.1230) with the *in vitro* synthesized glycated lysine. Similarly, glycated arginine (m/z = 337.1723) and glycated leucine/isoleucine (m/z = 294.1552) showed matching fragments (m/z = 60.0561, 70.0656, 114.0915, 173.1397, 217.1292, 257.1604) and (m/z = 86.0967, 132.1018, 230.1384, 258.1332) with their *in vitro* forms, respectively (Table 2, Figure 1, and Figure S2). Apart from these three amino acids, the glycated forms of other amino acids were not detected in the plasma. Diabetic plasma was used to detect glycated amines with the assumption that their abundance would be higher under the hyperglycemic conditions.

Besides glycated amino acids, glycated urea was detected in diabetic plasma. The precursor (m/z = 223.0930) and fragment masses (m/z = 104.9924, 116.9923, 135.0027) for monoglycated urea (MGU) from the diabetic plasma matched with the *in vitro* synthesized MGU (Table 2, Figure 2, and Figure S3A). Similarly, m/z = 385.1458 corresponding to DGU showed the following fragment matches (m/z = 203.0523) with synthetic DGU. (Table 2, Figure 2, and Figure S3B). The proposed reaction between glucose and urea leading to the formation of MGU and DGU is depicted in Figure 3A,B. Apart from glycated urea, the formation of glycated creatine was analyzed. However, we could not detect the glycated forms of creatine in the plasma.

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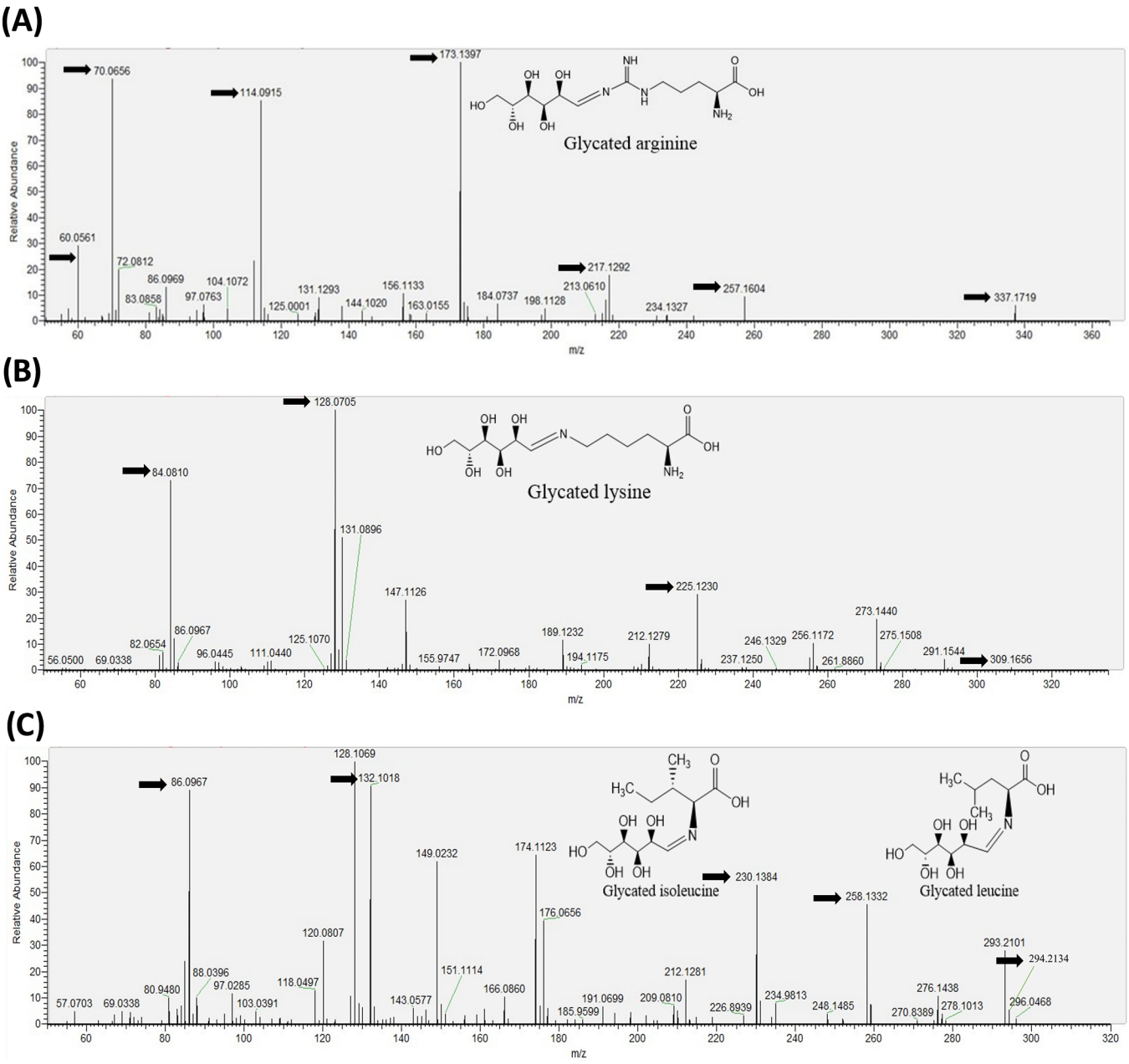

Figure 1. Mass spectrum of glycated amino acids derived from diabetic plasma samples (predicted structures are shown in the inset). Matching fragments are indicated with the arrows. (A) Detected glycated arginine from diabetic plasma samples, (B) detected glycated lysine from diabetic plasma samples, and (C) detected glycated isoleucine/leucine from diabetic plasma samples.

Quantification of MGU and DGU by PRM. Blood urea nitrogen is one of the diagnostic markers for kidney dysfunction and diabetic nephropathy.[9] Currently, urea is quantified enzymatically using urease, which may not detect glycated urea, thus affecting the accuracy of diagnosis. Therefore, in this study, MGU and DGU were quantified by PRM in healthy (n = 26), diabetic (n = 26), and diabetic nephropathy (n = 23) subjects. The mean peak area of MGU (8.8×10^5) was more abundant than the peak area of DGU (1.7×10^5) in all the subjects (Table S1). For normalization, $^{13}C_6$-MGU and $^{13}C_6$-DGU were synthesized using $^{13}C_6$-glucose and urea, and their mass spectral fragmentation was compared to $^{12}C_6$-MGU and $^{12}C_6$-DGU. The MS/MS spectra of $^{12}C_6$-MGU and $^{12}C_6$-DGU showed a similar fragmentation pattern to that of $^{13}C_6$-MGU and $^{13}C_6$-DGU, respectively (Figure S4A,B). Therefore, the peak area of *in vitro* synthesized $^{13}C_6$-MGU and $^{13}C_6$-DGU was used for normalization.

A representative Skyline generated peak area for MGU and DGU is depicted in Figure 4A,C, respectively. The normalized AUC values of MGU (mean ± SD) in healthy, diabetes, and DN were 1.01 ± 0.29, 1.62 ± 0.65, and 1.54 ± 0.76, respectively (Table S1). The normalized mean MGU area was significantly higher in DM and DN compared to healthy subjects. However, the MGU levels were not significantly different between those of DM and DN (Figure 4B). The DGU also showed a similar trend in H, DM, and DN. The normalized AUC of DGU was significantly higher in DM and DN (Figure 4D, Table S1). However, the values of DGU appear to be higher than those of MGU, although MGU was more abundant. This is due to the lower detection of $^{13}C_6$-

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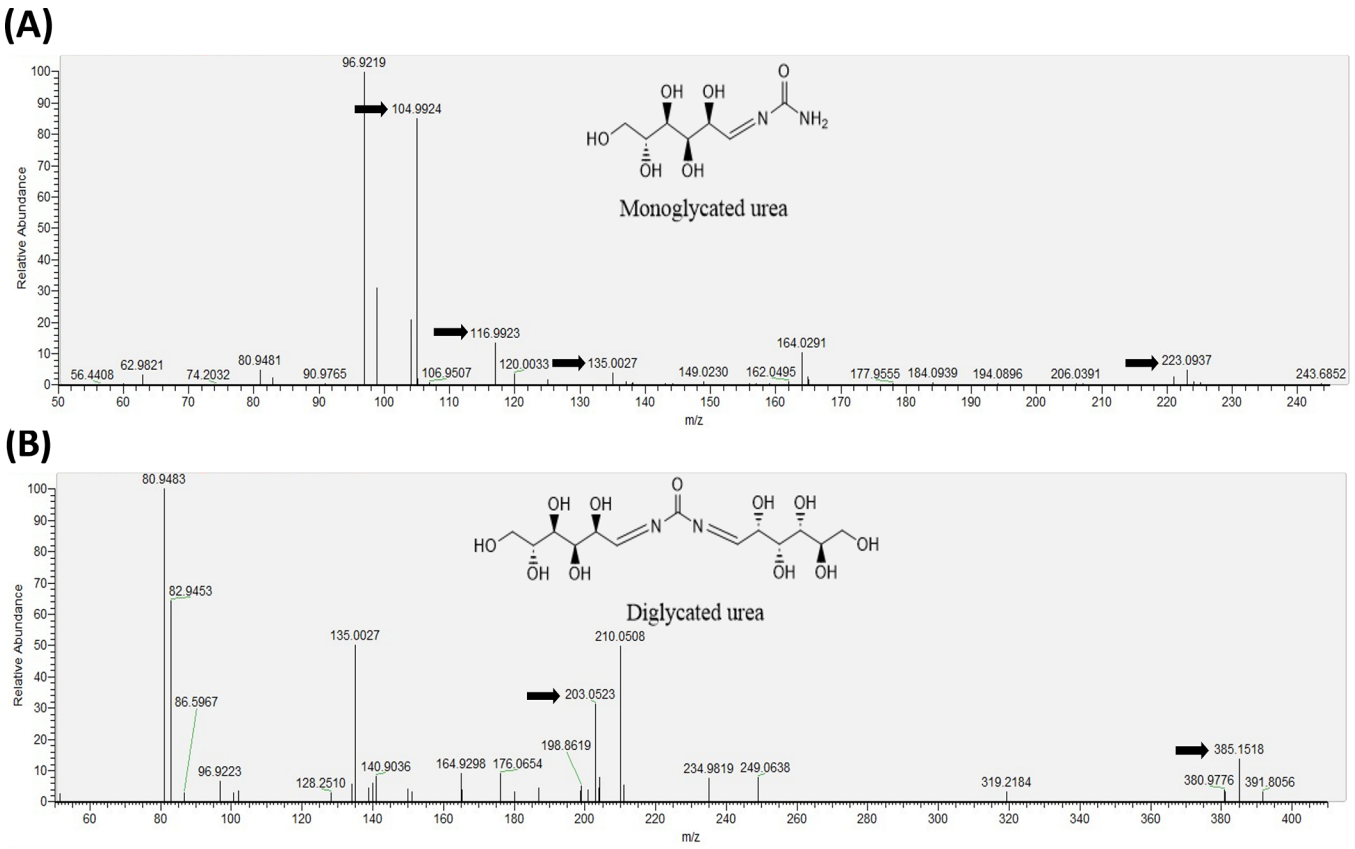

Figure 2. Mass spectra of (A) monoglycated urea (MGU) and (B) diglycated urea (DGU) from diabetic plasma. Matching fragments are indicated with the arrows.

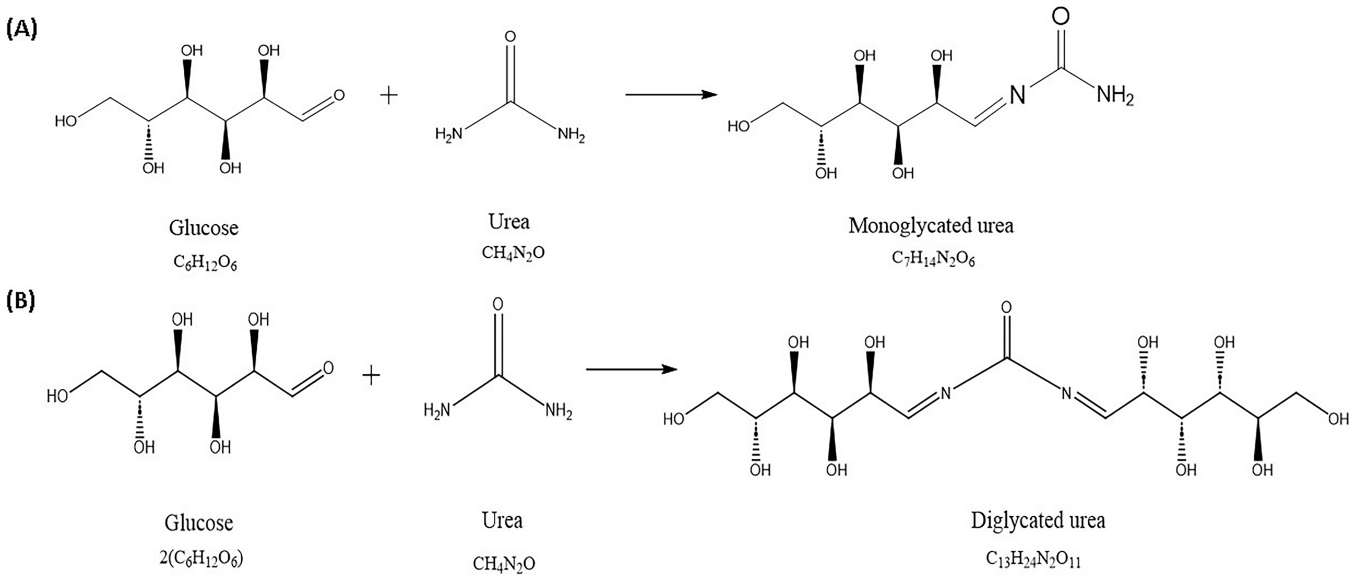

Figure 3. Proposed glycation reaction for (A) monoglycated urea (MGU) and (B) diglycated urea (DGU) synthesis.

DGU in the mass spectrometric analysis and its use in normalization.

Furthermore, the abundance of MGU and DGU was correlated with the various clinical parameters. Both MGU and DGU showed significant correlations with fasting blood glucose, postprandial glucose, and HbA1c. MGU showed a significant correlation with fasting blood sugar level (r = 0.78****), postprandial blood sugar level (r = 0.64****), and HbA1c (0.62****) (Table 3). DGU also showed a significant correlation with fasting blood sugar level (r = 0.74****), postprandial blood sugar level (r = 0.62****), and HbA1c (r = 0.58****) (Table 3; ****p <0.0001). However, both did not show a strong correlation with the known markers of kidney dysfunctions, such as microalbumin and serum creatinine.

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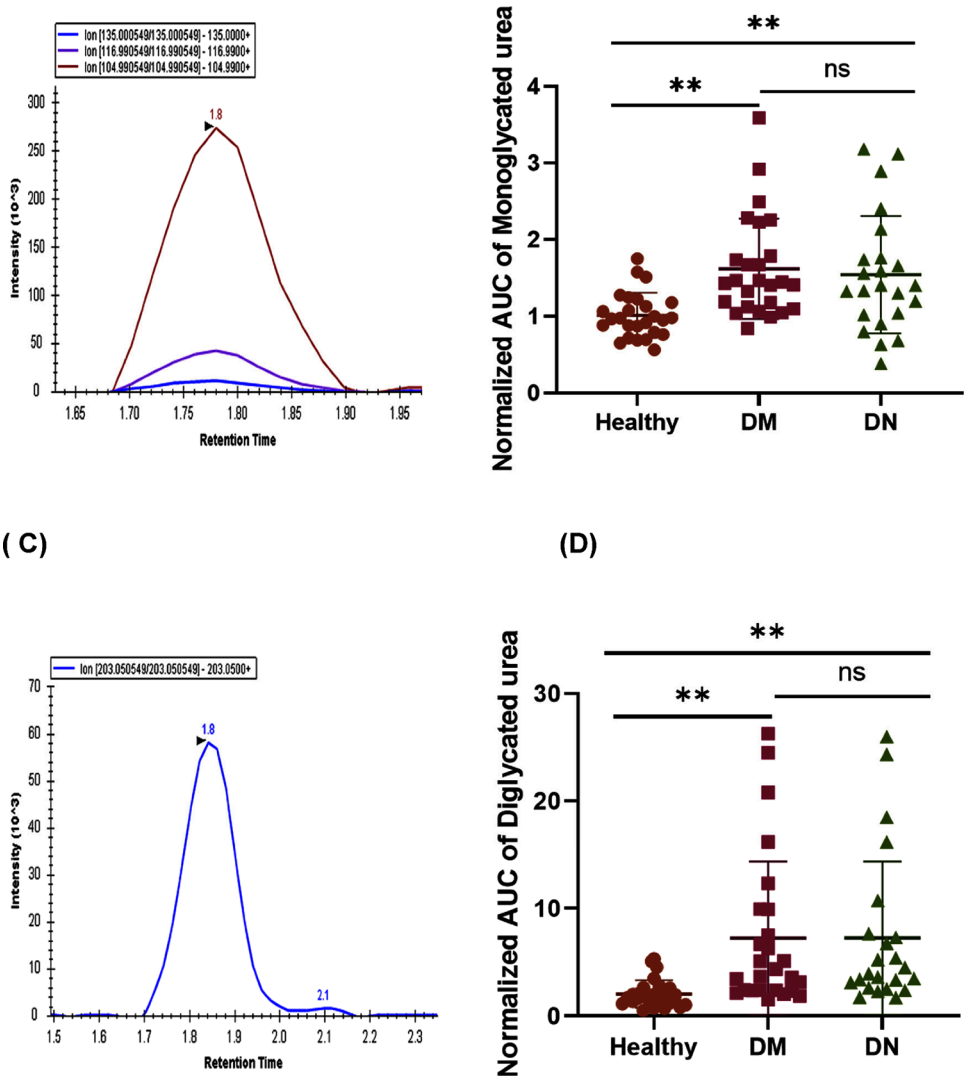

Figure 4. Representative chromatogram of (A) monoglycated urea (MGU) obtained from Skyline analyses. (B) Normalized AUC of MGU in healthy, diabetic, and diabetic nephropathy plasma samples. (C) Diglycated urea (DGU) obtained from Skyline analyses. (D) Normalized AUC of DGU in healthy, diabetic, and diabetic nephropathy plasma samples. (ns- non significant, *- signicant at p<0.05, ** -significant at p<0.01)

Table 3. Correlation of Glycated Urea with Clinical Parameters[a]

	MGU		DGU	
clinical parameters	*r*	*p*-value	*r*	*p*-value
HbA1c	0.62	<0.0001	0.58	<0.0001
FBSL	0.78	<0.0001	0.74	<0.0001
PP BSL	0.64	<0.0001	0.62	<0.0001
serum creatinine	0.004	0.97	−0.029	0.8
microalbumin	0.135	0.29	0.184	0.14

[a]FBSL: fasting blood sugar level, PPBSL: postprandial blood sugar level ****p <0.0001.

DISCUSSION

A wealth of information exists concerning the glycation of proteins in diabetes. However, the information on glycation of free small-molecule amines in humans is very scanty. In this quest, free glycated amino acids and glycated urea were discovered from diabetic plasma in this study. Glycated amines were synthesized *in vitro,* and a mass spectral library comprising precursor and fragment masses was developed. This information was used to identify free glycated amino acids and glycated urea from diabetic plasma. For the first time, we report the identification of glycated lysine, glycated arginine, and glycated leucine/isoleucine from diabetic plasma. The presence of these glycated amino acids in plasma was validated by comparing their precursor and fragment masses to those of the synthetic glycated amino acids. The discovery of free glycated amino acids has great physiological significance. Glycation of amino acids may create a condition akin to amino acid deficiency as the glycated amino acids may not be suitable to carry out the functions of amino acids. Amino acids are essential for protein synthesis, and their deficiency may affect protein synthesis. In diabetic conditions, there is an overall decline in protein content and muscle mass.[13] This decrease is attributed to increased protein catabolism due to an insulin deficiency. However, based on this study, we propose that the glycation of amino acids leads to the deprivation of amino acids and affects protein synthesis. Additionally, amino acids are involved in cell signaling and metabolic regulation. Amino acids activate the mTOR1 pathway, which in turn, through a cascade of signaling, induces insulin secretion. In contrast, the deficiency of amino acids activates GCN2, which inactivates

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the mTOR1 pathway, thereby affecting insulin secretion.[14] A decrease in insulin secretion may lead to hyperglycemic conditions and glycation of amino acids, thereby forming a vicious cycle of amino acid deficiency and activation of the GCN2 pathway.

Furthermore, glycation of lysine, arginine, and leucine/isoleucine under hyperglycemic conditions may create their deficiency and affect their function. Lysine is known for positive nitrogen balance, and its deficiency results in a decrease in body weight.[15] Lysine has antihyperglycemic effects and can improve diabetic conditions in humans and rats by reducing blood sugar and nonenzymatic glycation. Lysine lowers blood glucose, ameliorates nephropathy, and postpones cataracts in diabetic rats.[16] Besides its actions as a chemical chaperone, L-lysine can reduce inflammation by lowering the levels of interleukin 4 in the kidney and interleukin 10 in the liver.[17] Arginine, a functional amino acid, the precursor of nitric oxide, plays a crucial role in the maintenance, reproduction, growth, antiaging, and immunity of animals. It is synthesized from glutamate, glutamine, and proline via citrulline, whereas it is degraded to nitric oxide, ornithine, urea, polyamines, proline, glutamate, creatine, and agmatine through various pathways. In fact, a large amount of arginine is catabolized to creatine, which is a potent antioxidant and improves glucose tolerance.[18] Hyperglycemic conditions in ketosis-prone diabetes have been shown to be associated with decreased arginine availability and decreased insulin secretion, whereas exogenous arginine supplementation restored insulin secretion.[19]

Isoleucine/leucine are branched-chain amino acids (BCAA), essential for protein synthesis, involved in mTOR signaling, and a nitrogen source for glutamate, glutamine, alanine, and aspartate synthesis. The BCAA levels decrease in hyperammonemia states like liver cirrhosis and urea cycle disorders.[20] Although the levels of BCAAs are higher in diabetes, they can undergo glycation and may not be available for ammonia detoxification. BCAAs are also crucial for stimulating β-cell electrical activity, upregulating glucose transporters and insulin secretion, while glycation may affect these processes.

Apart from affecting key physiological functions due to glycation, these glycated amino acids can potentially serve as markers for diabetes and its complications. Diabetic nephropathy is one of the major diabetic complications. Currently, it is diagnosed by microalbuminuria, serum creatinine clearance, and blood urea nitrogen.[10] Creatinine is formed from dehydration and dephosphorylation of creatine.[21] Creatine is a small-molecule amine, can undergo glycation, and consequently may lead to lesser production of creatinine, thus underestimating creatinine accumulation in the serum. However, in this study, we could not detect glycated creatine in diabetic plasma. Similarly, blood urea nitrogen is also used to diagnose diabetic nephropathy.[10] During detoxification of ammonia, arginine is converted to ornithine and urea. Urea is a small-molecule amine with two primary amino groups and is susceptible to glycation modification under hyperglycemic conditions. In this study, for the first time, we report the discovery of the formation of glycated urea, which was detected in monoglycated and diglycated forms in diabetic plasma. The abundances of both monoglycated urea (MGU) and diglycated urea (DGU) were found to be higher in the plasma of diabetes and diabetic nephropathy. Both MGU and DGU were significantly correlated with fasting blood glucose, postprandial glucose, and HbA1c. Although neither MGU nor DGU showed a significant correlation with the markers of diabetic nephropathy, their formation may lead to an underestimation of the urea content in diabetic nephropathy. Therefore, quantification of glycated urea could be useful to detect early kidney disease. These conclusions were drawn based on a very small sample size (n = 75), which was the major limitation of this study. The usefulness of glycated urea as a marker of diabetic nephropathy needs to be established in a larger cohort study. Also, it is important to study the physiological role of glycated urea, including MGU and DGU accumulation, in diabetes. Additionally, it would be interesting to quantify glycated urea under uremic conditions. Apart from these, the other biogenic amines such as serotonin, tryptamine, ornithine, polyamines like spermine and spermidine, GABA etc., are also susceptible to *in vivo* glycation in hyperglycemic conditions. It would be interesting to study the effect of glycation on these molecules under diabetic conditions.

CONCLUSIONS

In this study, for the first time, we report the discovery of glycation of free lysine, arginine, and leucine/isoleucine from diabetic plasma. This has great physiological significance, as glycation of these amino acids may affect their vital functions such as protein synthesis, cell signaling, and insulin secretion. Also, glycated amino acids could be used as potential markers for the diagnosis and management of diabetes and its complications. Diabetic nephropathy is one such complication, where amines, such as creatinine and urea, accumulate in the plasma. For the first time, we report the detection of glycated urea in diabetic plasma. Further, we quantified MGU and DGU by a targeted mass spectrometric approach in the plasma of healthy, diabetes, and diabetic nephropathy subjects. Both MGU and DGU show a strong correlation with clinical parameters, such as blood glucose and HbA1c. Given that urea gets converted to glycated urea under hyperglycemic conditions while quantifying urea for kidney diseases, glycated urea needs to be accounted for.

ASSOCIATED CONTENT

Data Availability Statement

All the mass spectrometry acquisition raw files are stored on the Metabolights server (https://www.ebi.ac.uk/metabolights) with data set identifier MTBLS9851.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c01772.

(Figure S1) MS/MS spectra of *in vitro* synthesized glycated amino acids, (Figure S2) MS/MS spectra of glycated amino acids detected in diabetic plasma and their corresponding *in vitro* synthesized glycated amino acids with the matching fragments, (Figure S3) MS/MS spectra of glycated MGU and DGU detected in diabetic plasma and their corresponding *in vitro* synthesized MGU and DGU with the matching fragments, (Figure S4) MS/MS spectra of $^{12}C_6$ MGU and $^{13}C_6$ MGU and MS/MS spectra of $^{12}C_6$ DGU and $^{13}C_6$ DGU, and (Table S1) detailed information on cumulative mean and normalized mean in all subjects (PDF)

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Author Contributions

#R.Q.S. and S.D. had equal contribution.

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R.Q.S.: data curation, formal analysis, investigation, methodology, writing—review and editing; S.D.: data curation, formal analysis, investigation, methodology, writing—review and editing; A.C.: formal analysis, investigation, methodology, writing—review and editing, validation, visualization; M.G.A.: formal analysis, investigation, validation, writing—review and editing; A.B.S.: writing—review and editing, validation; M.F.: writing—review and editing, validation; S.T.: investigation, resources writing—review and editing; A.G.U.: investigation, resources, writing—review and editing; M.J.K.: conceptualization, investigation, methodology, project administration, supervision, writing—original draft.

Notes

The authors declare no competing financial interest.

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