Chapter

Individual Glycation Sites as Biomarkers of Type 2 Diabetes Mellitus

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Abstract

Type 2 diabetes mellitus (T2DM) is a widely spread metabolic disease, the initial stages of which are asymptomatic and have no clinically recognizable manifestation. At the molecular level, T2DM is manifested with essential non-enzymatic structural changes of intra- and extracellular proteins, mostly represented with oxidation and glycation of multiple residues. Protein glycation is one of the most universal markers of T2DM, and is recognized as an indirect, but adequate indicator of plasma glucose levels over prolonged periods of time. Unfortunately, glycated hemoglobin (HbA1c) – the universally accepted T2DM marker, is insensitive for short-term excursions of blood glucose, which are known to precede the onset of disease. Therefore, new generation biomarkers, giving access to the time dimension of Maillard reaction in blood, are desired. In this context, establishment of individual glycation sites of plasma proteins as new T2DM biomarkers might be a promising approach. Indeed, involvement of proteins with different half-life times in such analysis will make the time dimension of protein glycation in blood available and will allow early recognition of blood sugar fluctuations, occurring within few weeks or even days.

Keywords: Amadori compounds, biomarkers, glycation, glycation sites, label-free quantification, mass spectrometry, plasma proteins, stable isotope-labeled peptide standards, type 2 diabetes mellitus (T2DM)

1. Introduction

T2DM is featured with late onset of the pathology, and represents the form of diabetes which is characterized by insulin resistance, high level of blood sugar, decreased insulin production and pancreatic β-cell deficiency [1]. This state is typically accompanied with suppression of the glucose transport in muscle cells, hepatocytes and adipocytes [2]. Moreover, the increase of blood glucose is accompanied with enhanced degradation of lipids, especially triacylglycerols [3]. Various factors such as hyperglycemia and associated glucotoxicity, lipotoxicity, oxidative stress and mitochondrial dysfunction can induce apoptotic death of ß-cells [4]. As a result of β-cell dysfunction, the fasting-related secretion of the hormone glucagon and the levels of glucose in hepatocytes are not suppressed by subsequent food intake [5]. According to the World Health Organization (WHO), T2DM accounts for 90–95% of the overall number of diabetes cases and its complications are

recognized as the important factors causing blindness, kidney failure, heart attacks, stroke and lower limb amputation [1]. The disease is widely distributed in all parts of human population, and occurs in all regions, including rural areas of low- and middle-income countries. China, India, USA, the countries of Middle East and North Africa are in the top of diabetes occurrence rates [6]. The number of diabetes cases is growing steadily. According to the estimations of the International Diabetes Federation, the occurrence of diabetes will increase from approximately 463 million adults (20–79 years) in 2019 to 700 million by 2045 [7].

The onset of T2DM and its early stages are asymptomatic, as well as a period preceding T2DM and characterized with compromised regulation of blood glucose concentration (so-called pre-diabetes) [8]. Because of this, pre-diabetes is asymptomatically changed by T2DM, and this disease is usually diagnosed at relatively advances stages, when therapy is already required. Thus, to date, approximately every second T2DM case (232 million people) remain undiagnosed [7, 8]. Therefore, timely identifying the individuals in the pre-diabetic state, i.e. with the obvious high risk of developing T2DM is critically important, since early interventions can delay or even prevent onset of the full-scale disease manifestation [9].

Glycation, also often referred to as non-enzymatic glycosylation, represents a reversible reaction of amino and guanidino groups in proteins, peptides, and lipids with reducing sugars (aldoses and ketoses) and carbonyl products of their degradation [10]. The process of protein glycation is often referred to as Maillard reaction of proteins, i.e. the knowledge on its chemistry goes back to the works of Louis Camille Maillard in 1912 [11]. In this reaction, the carbonyl groups of reducing sugars interact with amino/guanidino functions of proteins (mainly with lysine, arginine residues and with N-terminal amino acid residues), with lipids and nucleic acids, yielding early glycation products, also known as Amadori [12] and Heyns compounds [13]. These compounds, often referred to as early glycation products, readily undergo rearrangement, cross-linking, oxidative and non-oxidative degradation, forming so-called advanced glycation end products (AGEs), which are known to accompany not only diabetes complications, but also neurodegenerative diseases and aging [14].

Currently, the Maillard reaction of proteins is considered to be the one of the most common pathways in formation of AGEs (**Figure 1**). Thereby, glycoxidation,

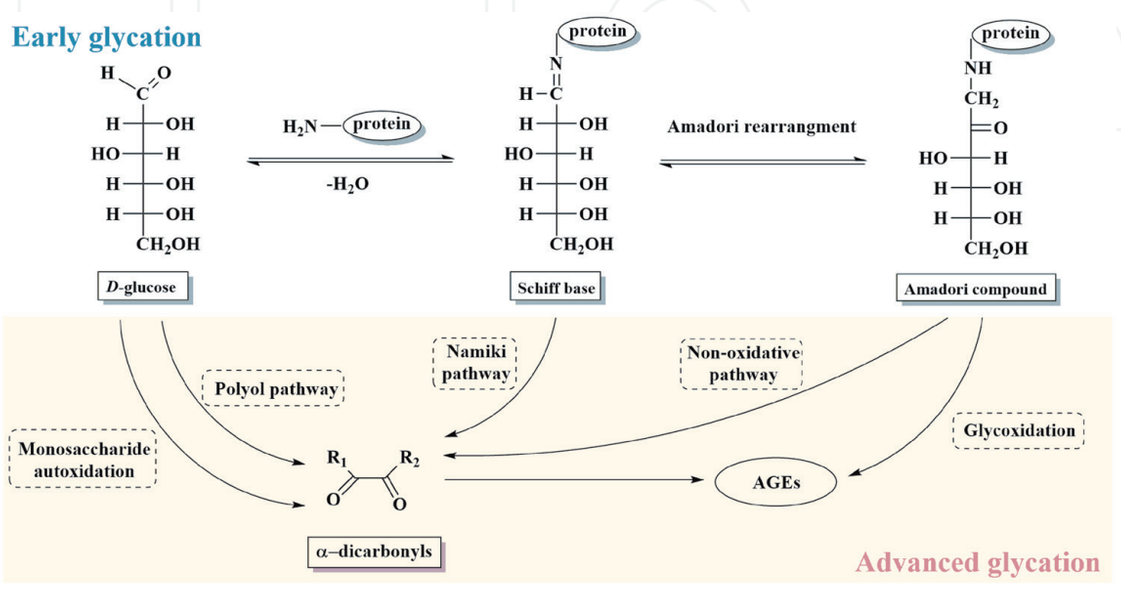

Figure 1.
Formation of early- and advanced glycation in human blood plasma.

i.e. oxidative degradation of early glycation products [15] represents the main route of AGE formation, at least for such derivatives as CML and pentosidine [16]. Importantly, α-dicarbonyl compounds, such as 3-deoxyglucosone (3-DG), glyoxal (GO), and methylglyoxal (MGO) are the principle intermediates of AGE formation [17]. Besides glycoxidation, these compounds can also appear as intermediates of glucose autoxidation [18], lipid peroxidation [19], polyol pathway [20], and the Namiki pathway [21]. These α-dicarbonyls are highly relevant for diabetes pathology, as increased concentrations of glyoxal, methylglyoxal, and 3-deoxyglucosone have been found in patients with T2DM [22]. Thus, interaction of GO with amino function yields N^{ε}-(carboxymethyl)lysine (CML), which represents one of the major and well-characterized AGE. Further cross-linking of GO-derived AGEs with another lysyl residue yields glyoxal-derived lysine dimer (GOLD) or arginine residue forming glyoxal derived imidazolium cross-link (GODIC) [23]. The GO-derived modification of arginine - glyoxal-derived hydro-imidazolinone (Glarg) slowly hydrolyzes under physiological conditions to yield acid-labile N^{δ}-(carboxymethyl)arginine (CMA) [24]. The interaction of MGO with lysine leads to the formation of N^{ε}-(carboxyethyl)lysine (CEL), and reaction with arginine produced argpyrimidine or methylglyoxal-derived hydro-imidazolone (MG-H1) [17]. The methylglyoxal-lysine dimer (MOLD) is one of the major non-enzymatic cross-links in serum proteins detected during metabolic disorders [25]. Interaction of 3-DG with lysyl residues yields such modifications as pyrraline, pentosidine, imidazolone or CML [26].

In human organism, AGEs exert clearly deleterious effects [27], which are manifested by changes in structure and functions of proteins in human blood. AGEs can interact both with individual proteins *(per se)*, and by direct binding to them through the formation of cross-links. Glycation products are often found in extracellular tissue structures, thus, modified proteins impair matrix–matrix and matrix-cell interactions, leading to reduced cell adhesion, migration and cell death [27]. Intracellular proteins are also readily involved in a broad array of modifications and might lose their functionality at least to some extent [28]. The negative effects of AGEs are summarized on the **Figure 2**.

The term "fructosamine", is a common definition for all ketoamine-containing substances, i.e. Amadori and Heyns compounds, formed during protein glycation [29]. Glycated hemoglobin, which has a lifespan of 90–120 days [29], is routinely used for monitoring of glycemic index in blood of individuals with both T1DM and T2DM. Accumulation of the minor hemoglobin isoform HbA1c, also often referred to as glycated hemoglobin, in blood of diabetic patients was first reported in 1968 [30]. The modification of hemoglobin, underlying this isoform, occurs at the N-terminal valine residue of the β-chain and yields a fructosamine adduct (Amadori product) with high diagnostic value [31].

To date, more than 300 different analytical methods and well-established assays for quantitative determination of HbA1c are reported. The most of these methods rely on ion-exchange chromatography (IEC), high-performance liquid chromatography (HPLC) [32], boronate affinity chromatography (BAC) [33], colorimetry [34], as well as different biosensors, based on amperometric [35], potentiometric [36], impedometric [37] and optical sensing [38] techniques. It needs to be taken into account, however, that in some cases determination of HbA1c may appear to be biased or even unreliable. Such cases include pregnant women and patients with end-stage renal disease or those suffering from heavy alcohol consumption [29]. The other well-recognized limitations of the method are its compromised performance with the individuals characterized with increased red blood cell turnover (the state accompanying, for example, hemolytic anemia and severe blood loss) and interference between different hemoglobin isoforms variants [29].

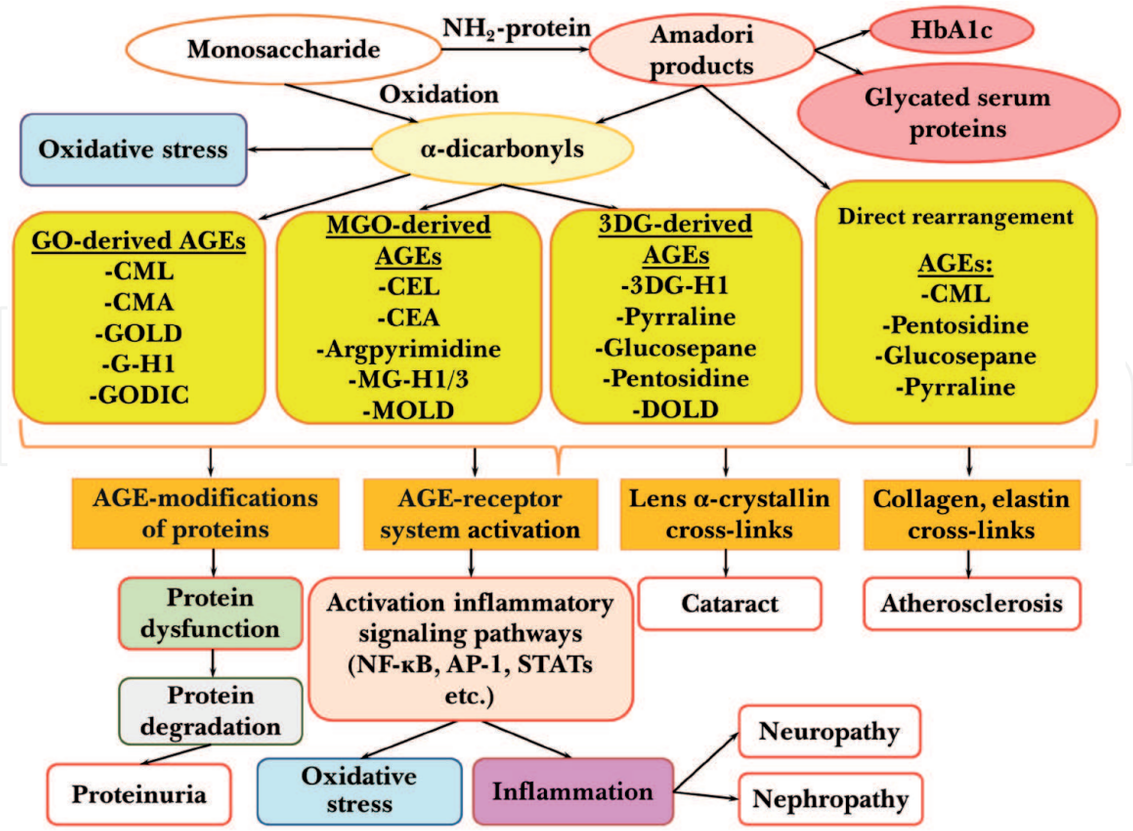

Figure 2.
Pathological role of AGEs in diabetic complications. 3-deoxyglucosone-derived hydroimidazolone 1 (3DG-H1); activator protein-1 (AP-1), N^{δ}-(carboxyethyl)arginine (CEA), 3-deoxyglucosonederived lysine dimer (DOLD); glyoxal-derived hydroimidazolone 1 (G-H1), nuclear factor k-light-chain-enhancer of activated B cells (NF-kB), signal transducer and activator of transcription (STAT).

To some extent, these limitations can be addressed by implementation of a highly abundant plasma protein as a glycation biomarker supplementary to HbA1c. Human serum albumin (HSA) –the major plasma globular polypeptide with a molecular weight of approximately 67 kDa and a serum half-life of about 20 days, is recognized as the best candidate for such kind of biomarker since decades [39]. HSA constitutes up to 70% of the total serum protein, being the most abundant polypeptide in blood plasma [40]. Thus, glycated HSA (often also referred to as glycated albumin, GA) can be employed in determination of glycemic status when the conventional marker (HbA1c) is not reliable enough [41].

Application of GA can be advantageous in comparison to HbA1c due to lower reagent cost and the ability to automate GA analysis on many common laboratory instruments [29]. To date, multiple methods we proposed for analysis of GA. These include enzymatic assays [42], IEC-HPLC [43] and two-dimensional liquid chromatography including affinity chromatography and separation on reversed phase [44]. Immunoassays also represent a promising approach for assessment of GA contents. This strategy can be implemented by radioimmunoassay [45], ELISA [46], enzyme-linked boronate immunoassays (ELBIA) [47], colorimetry [48] and electrochemical methods [49]. Method characteristics are summarized in the **Table 1**.

Among the strategies of GA analysis, enzymatic method characterized by shorter operational time and easier performance both in manual and automatic mode [54]. This approach relies on exhaustive hydrolysis of GA by albumin specific proteinase with subsequent oxidation of resulted glycated amino acids by ketoamine oxidase to form hydrogen peroxide, which interacts with chromogen. The colored product can be quantified spectrophotometrically at 546/700 nm. The contents of GA are expressed as the percentage of glycated albumin in total

#	Technique	Protein isolation	Detection	Value of GA	Ref
1	Radioimmuno-assay	Precipitation (ice-cold trichloroacetic acid solution)	Gamma irradiation	Control subjects: 2.0 ± 0.24 nmol/mg, T2DM: 5.3 ± 2.8 nmol/mg	[45]
2	ELISA	Affinity chromatography	UV: absorbance at 450 nm	Control subjects 2.4 ± 0.22%. Diabetic patients: 4.5 ± 1.2% (1.6–11.6%)	[50]
3	ELBIA	Affinity chromatography	UV: absorbance at 492 nm	Control and diabetic subjects: 1.1% - 47.8%	[47]
4	Enzymatic assay	—	UV: absorbance at 546/700 nm	Control subjects 13.4% (range 11.7–16.9%). T2DM 17.4% (14.2–27.0%) in good control and 26.4% (22.6–49.9%) in poor control	[51]
5	Colorimetry	Fractionation with polyethylene glycol	UV: absorbance at 546/700 nm	Control subjects: 160–222 (μmol/L). Diabetic patients: 424.6 ± 83.6 μmol/L in T1DM and 346.5 ± 61.6 μmol/L in T2DM	[48]
6	IEC-HPLC	Anion exchange chromatography, boronate affinity chromatography	UV: excitation wavelength 285 nm, emission wavelength 340 nm	Control subjects 20.2 + 1.6% (range 17.2–23.4%). Diabetic patients: 39.6 + 5.4% in T1DM and 39.4 + 5.9% in T2DM	[43]
5	Electrochemistry	—	Electro-chemical aptasensor	Control subjects 2–4%. Diabetic patients ≥16%	[52]
6	Lateral flow immunoassay	—	Colori-metric detection	Control subjects 4.59 ± 0.66 mg/mL (2.44–5.55 mg/mL). Diabetic patients 7.16 ± 2.58 mg/mL (3.17–17.21 mg/mL)	[53]

Table 1.
Overview of analytical techniques employed in analysis of GA. Ultraviolet detector, UV.

albumin [42]. Although this method was proposed more than 15 years ago, it allows easy and fast quantification of GA with good analytical performance (specificity, accuracy, reproducibility) [55–57]. However, enzymatic assays usually require high concentration of HSA in samples that essentially restricts applicability of the method. Recently, an improved lateral flow immunoassay (LFIA) for simultaneous colorimetric determination of the total HSA and GA, which mostly solves this problem [58].

Due to the wide range of polarities and the different structure of AGEs they can be analyzed by a variety of techniques including spectrofluorimetry, enzyme-linked immunosorbent assays (ELISA), HPLC with UV–VIS detection or coupled on-line to mass spectrometry (MS) [59]. The lack of standardized methods and reference materials increases the risk of analytical errors, negatively affecting accuracy and reproducibility of these methods. Because of this the analysis of AGEs is not widely spread in regular clinical practice [60].

Analysis of total plasma contents of Amadori compounds represents another strategy of glycemic control. Determination of total blood fructosamines provides information on glucose control in the time-frames, mostly limited to the previous two weeks [61]. The total plasma fructosamine content was for the first time used as a diabetes marker in 1983 [62]. The corresponding analytical method relied on the reduction of the dye nitroblue tetrazolium (NBT) to formazan. The level of formazan formation is directly proportional to the fructosamine concentration and can be then assessed by spectrophotometry [62]. The method was significantly improved in 1989 by supplementation of incubation mixtures with a non-ionic detergent in combination with uricase. This made it possible to eliminate the influence of uric acid, lipaemia and polylysine and to provide better sensitivity [63, 64]. Although the method has some disadvantages, such as temperature sensitivity, interference with potential inhibitors of response (such as vitamins and bilirubin) and low standardization, today it remains a valuable tool, which is characterized with ease of handling, low coasts and high potential for automation [29].

Due to a high structural diversity of AGEs their adequate analysis represents a challenging task. In the most easy and straightforward way, analysis of AGEs can rely on their spectral properties, which give access to the total AGE fraction. Indeed, generally, AGEs can be divided into two groups: fluorescent (such as pentosidine or glucosepane) and non-fluorescent AGEs (for example, CML and CEL) as shown on **Figure 3**. Therefore, due to the presence of fluorescent AGEs in the protein structure it is possible to assess AGE-specific fluorescence in serum, urine and saliva using the methods of spectrofluorometry [60]. This AGE-specific fluorescence of cross-linked AGEs can be detected at 440 nm after excitation at 370 nm [65]. For example, using this method, Villa et al. have shown a correlation

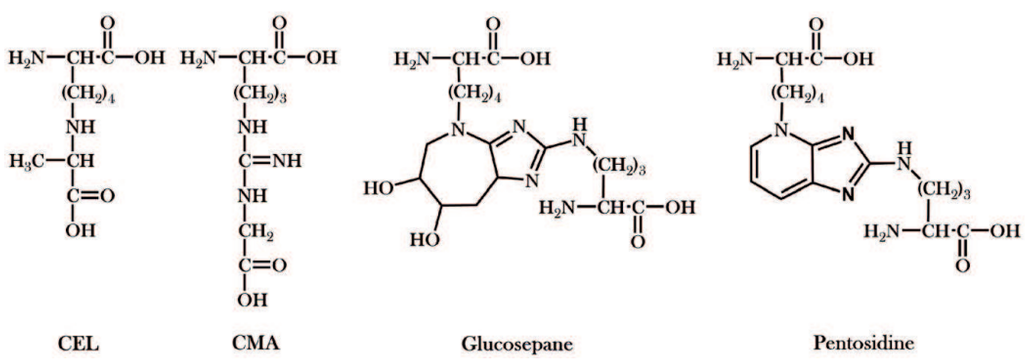

Figure 3.
Fluorescent (glucosepane, pentosidine) and non-fluorescent (CEL, CMA) AGEs; CEL, N^{ε}*-carboxyethyl lysine; CMA,* N^{ω}*-carboxymethylarginine.*

between *in vitro* glycated BSA, and the levels of circulating and tissue AGE in diabetic rats [66]. Although this method is simple, fast and cheap, there are some serious limitations, such as lack of detection of non-fluorescent AGEs and interference with non-AGE fluorophores [60]. Furthermore, since more than fifteen years a non-invasive method for *in vivo* determination of AGE-specific autofluorescence is established [67]. However, the presence of endogenous fluorescent signals from cutaneous fluorophores (e.g. nicotinamide adenine dinucleotide, NAD) having the same excitation and emission ranges can interfere with the correct measurement of total fluorescence [68].

Unfortunately, such a powerful method as ELISA is also not free from some intrinsic limitations. Thus, as it can rely not only on monoclonal, but also polyclonal primary antibodies [69], ELISA is often featured with insufficient specificity of antibodies. Moreover, it can suffer from such factors, as high background responses due to significant contents of protein glycation adducts [70] and interference with non-glycated modified or non-modified amino acid residues [71] due to heating and alkaline treatment, implemented in the protocol [72]. Enzyme-linked boronate-immunoassay (ELBIA) represents an efficient extension of ELISA, applicable, however, only to analysis of early glycation products. This technique was first established in 1998 as a method based on the interaction of boronic acids and cis-diols of glycated HSA captured by an anti-HSA antibody [47]. A fully automated ELBIA system, giving access to high-throughput, rapid and precise measurements of GA was also developed, which was an essential extension of the method.

It is known since decades, that individual AGEs can be used as biomarkers of different pathologies including diabetes itself. Thereby, individual AGE classes present in biological samples can be assessed by instrumental and immunochemical methods, which need to be more specific due to the targeted character of the analysis. These methods include RP-HPLC, coupled on-line to spectrofluorometry [73], mass spectrometry (MS) [74, 75] or tandem mass spectrometry (MS/MS) [22, 76, 77], as well as gas chromatography - mass spectrometry (GC–MS) [78]. Immunochemical methods are mainly ELISA and Western blotting, using antibodies specific for certain AGE structures [60]. Thus, CML and pentosidine significantly increased in patients with renal failure compared to control subjects [79] and T2DM compared to non-diabetic controls [80].

2. Identification of individual glycation sites

Like any chronic pathology, diabetes can be efficiently recognized by a set of reliable well-established methods according universal criteria [81]. However, its first manifestations are often invisible for patients and recognized, therefore, already after onset of the pathology [81]. Thus, early diagnosis of T2DM and timely start of its therapy would allow deceleration of the disease progress and reduction the probability of life-threatening complications. Therefore, it is very important to develop a panel of biomarkers, giving access to the early and reliable discovery of diabetes mellitus.

Although HbA1c, fasting blood glucose and glucose tolerance test are well established and universally recognized diagnostic criteria of DM [81], this setup is usually unable to recognize the short term excursions of blood glucose concentrations, which are characterized the beginning of pre-diabetes [82]. Therefore, it was proposed that the biomarkers based on disease-related structural changes of individual proteins might be more sensitive and, hence, more diagnostically efficient [82]. Among such changes, post-translational modifications (PTMs) represent the most promising source of diagnostic information [82–84]. Thereby, modified

peptides, rather than proteins represent the best targets in the search for new T2DM biomarkers of this type. Indeed, under *in vivo* conditions proteins can have multiple modification sites, the patterns of which can be very heterogeneous in terms of diversity of chemical structures (phosphorylation, nitration, carbonylation, glycosylation, methylation, acetylation and many others) and their relative abundances [85]. On the other hand, each individual PTM changes the molecular weight of the target protein, which leads to difficulties in MS analysis [86].

Superior, in comparison to protein analysis, level of precision could provide the fact, that individual lysine and arginine residues in protein molecule are featured with different reactivity with sugars. This effect can be related to both the amino acid environment of the site [87] and its accessibility to the molecule of glycation agent [86, 88]. Moreover, as the blood plasma proteins have different half-lives these markers can potentially cover a broad range of times prior to analysis. Thus, in contrast to HbA1c analysis, this approach might provide a short-term markers, which could successfully address short-term fluctuations in blood glucose, preceding onset of DM [84, 89]. Monitoring of blood protein glycation in this way might provide an opportunity for detection of hyperglycaemia at very early stages of the T2DM [90].

Since decades, the bottom-up proteomics (BUP) approach is the method of choice to address PTMs in proteins [91]. Accordingly, it is efficiently applied to analysis of protein glycation and can be applied to protein mixtures of any composition and complexity [92]. In the most general way it includes several critical steps: (*i*) separation of proteins, (*ii*) limited proteolysis, (*iii*) separation of resulted cleavage peptides, (*iv*) their identification by tandem mass spectrometry (MS/MS) and (*v*) annotation of individual protein sequence tags [79, 83, 89]. In application to sugar-modified proteins, BUP used for detailed information about glycoprotein profile and mapping of specific glycation sites [93].

For the BUP only several microliters of blood plasma are necessarily [83, 84]. The short workflow is present on the **Figure 4**. Plasma proteins can be separated during electrophoresis with further in gel digestion [94], or Amadori-modified proteins can be retained on BAC before digestion *in solution* [89, 92]. Several important aspects need to be continuously considered on this way. Thus, for successful quantitative BUP analysis it is very important to use the same concentration of protein in all samples [79, 80, 95]. Further, tryptic digestion of plasma samples is challenging because of high complexity of sample matrices and needs to be performed in the presence of chaotropic agents like urea [96] or detergents, e.g. sodium dodecyl sulfate (SDS) [83]. Next step is enrichment of glycated peptides on BAC which helps to eliminate chaotropic agents [83, 84, 97]. The BAC method is based on covalent binding of the column-bound ligand (*m*-aminophenyl-boronic acid) to cis-diol groups on the sugar portion of peptides, accompanied with formation of a reversible five-member ring derivative. After washing out non-bound unglycated molecules from the sample by alkaline buffer, the five-member ring can be hydrolyzed under acidic conditions, and glycated peptides can be eluted by acidic (pH 2–3) buffer [98, 99]. Prior to the MS analysis, the obtained peptides need to be desalted by solid phase extraction (SPE) [83, 84, 100, 101]. Several separation steps (on a protein and/or peptide level prior to separation by mass-to-charge ratio) and high specificity of endoproteases used for digestion are provide high proteome discovery rate and sensitivity [85, 87].

The gel-based strategy was implemented for analysis of glycation of apolipoprotein A-I in human plasma samples [94]. For this, blood samples were obtained from ten T2DM patients, affected by end-stage renal disease (ESRD), and ten healthy control individuals. The plasma samples were pooled by mixing the samples of each group of subjects and then applied onto a Centriplus centrifugal concentrator

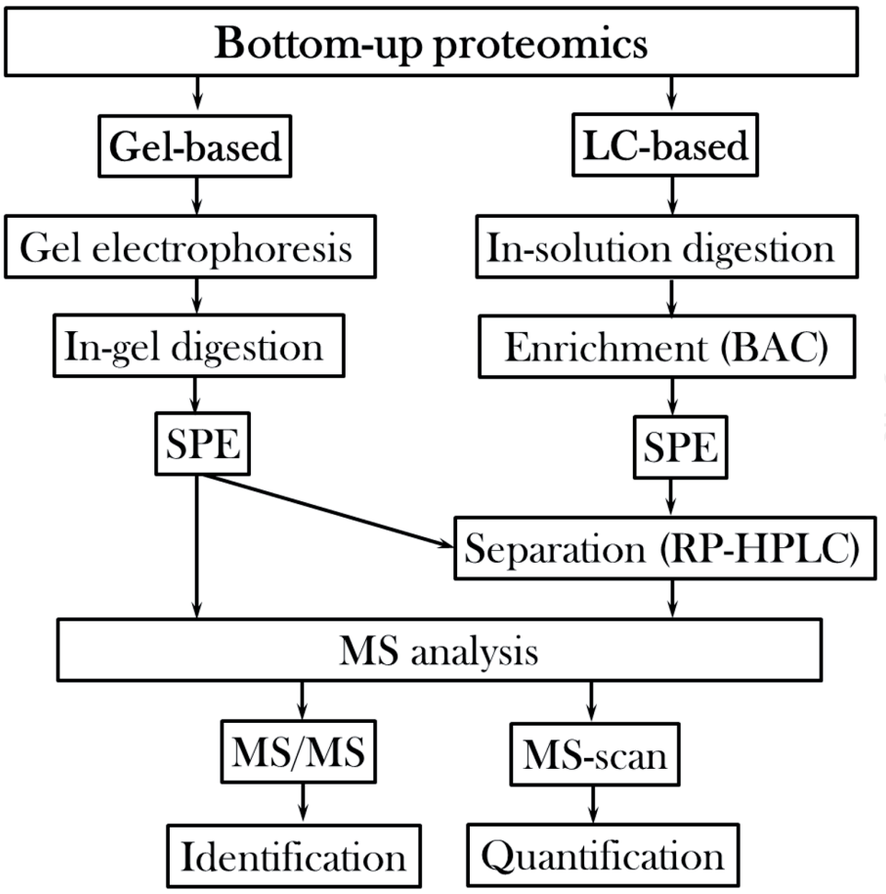

Figure 4.
The short workflow of analysis individual glycation sites.

membrane with molecular weight cut off (MWCO) 30000. After two-dimensional gel electrophoresis (2-DE) the apolipoprotein A-I spots were cut, digested, and the digests were analyzed by matrix laser desorption ionization time-of-flight (MALDI-TOF) with a standard nitrogen laser (λ = 337 nm). In this study three glycated peptides from apolipoprotein A-I were identified in T2DM and nephropathic patients [94].

One of the first scientific groups started developing methods for analysis of individual glycation sites in proteins of human plasma was the Metz's laboratory. Initially, they investigated *in vitro* glycated proteins in pooled plasma from healthy humans [96]. Glycated proteins were enriched using BAC and then digested by three different proteolytic enzymes (trypsin, Arg-C and Lys-C) to increase sequence coverage. After protein digestion, Amadori-modified peptides were enriched by BAC and analyzed by linear ion trap – orbital trap mass spectrometer (LIT-Orbitrap-MS) with electron-transfer dissociation (ETD) fragmentation option. As a result, 346 unique glycated peptides were identified. It was shown that trypsin was the most applicable enzyme in study of glycated peptides [96].

Alternatively, Zhang et al. performed the first proteomics-based characterization of non-enzymatically glycated proteins in human plasma and erythrocyte membranes from participants with normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and T2DM [102]. In this study one additional step was introduced, and twelve highly-abundant plasma proteins were removed from the samples during immunodepletion procedure. Depletion of such proteins as HSA, immunoglobulin G (IgG), α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α1-acid glycoprotein, α2-macroglobulin, apolipoprotein A-I, apolipoprotein A-II and fibrinogen from blood plasma enabled the analysis of less abundant plasma

proteins. As the result, 260 unique Amadori-modified peptides representing 76 unique glycated proteins from human plasma were identified. Among them 39 unique glycated proteins, represented by 114 unique glycated peptides could be detected in human plasma prior to immunodepletion. On the other hand, further 46 unique glycated proteins (156 unique glycated peptides) were discovered in the low-abundance protein fraction of human plasma. As for the proteins of the erythrocyte membrane, 75 unique glycated peptides corresponding to 31 unique glycated proteins were identified. That means, that under diabetic conditions the functions of major structural proteins, major integral proteins of erythrocyte lipid rafts and GAPDH are affected by glycation. Interestingly that a majority of the identified Amadori-modified proteins appear in all three subject groups, with little variation in terms of the numbers of glycated peptides or glycation sites. In that study no label-free-quantification analysis was performed. However, a roughly estimation showed, that 50 of unique glycated peptides from plasma samples and 14 from erythrocyte membrane were up-regulated in both IGT and T2DM groups compared to the NGT group [102].

The next logical step of the Metz's work was comprehensive identification of glycated peptides in plasma and erythrocytes of control and diabetic subjects performed in 2011 [87]. After a three-step separation by strong cation exchange chromatography (SCX), BAC, nanoHPLC and sub-sequent mass spectrometric analysis with ETD-based fragmentation, a comprehensive database of glycated peptides/glycation sites and corresponding proteins was built to facilitate the discovery of potential novel markers of diabetes. For selective and specific identification of glycated peptides, the authors established a data-dependent neutral loss triggered ETD scan, where the top six most intense ions were first fragmented with and precursor ions producing neutral losses of 3 H2O and 3 H2O + HCHO (characteristic neutral losses for Amadori-modified peptides during CID [103]) were further fragmented using ETD. In total, 7749 unique glycated peptides corresponding to 3742 unique glycated proteins were identified [87], that was a massive advantage in sequence coverage in comparison to the previous study. In general, characteristic neutral losses represent a convenient and powerful tool in identification of glycation products: they allow not only identification of involved monosaccharide [99, 100], but also more complex modifications, like ADP-glucose-dependent glycation [104].

In the work of Bai *et al.* [95] the analysis of glycated HSA peptides by liquid chromatography – ion trap – time-of-flight (LC-IT-TOF)-MS revealed 21 glycation sites in the serum samples of healthy persons and only 16 glycation sites in that from the T2DM patients [95]. Here BAC was used for enrichment of glycated proteins. The sub-sequent digestion procedure was carried out by incubation with endoproteinase Glu-C and trypsin. High sequence coverage (88% for GA from healthy person and 78% for GA from T2DM) was achieved by combining the peptide mass fingerprinting mapping results of the digests, obtained by both Glu-C and trypsin [95].

Using capillary flow data-independent acquisition (DIA) proteomics approach, 234 glycation sites in human plasma proteins were characterized [100]. 1508 plasma samples were obtained from overweight/obese non-diabetic adults. For DIA analysis, peptides were loaded on RP-UHPLC coupled on-line to Orbitrap Fusion Lumos MS tribrid. Full MS scan was performed from 350 to 1650 *m/z*, then 33 DIA segments were acquired with higher-energy collisional dissociation (HCD) 27%. For DDA analysis, isolation width was set to 1.6 *m/z*, 3 s method cycle time and 27% HCD for the dependent MS/MS scans. It resulted in identification of 242 glycation sites on 70 proteins. In this study most glycation sites were detected in serum albumin (36 sites), serotransferrin (13) and Ig kappa constant region (7) [100].

AGE-modified sites were also in the focus of research groups working in the field of DM biomarker discovery. Greifenhagen *et al.* [105] optimized the method for identification carboxymethylated (CML-modified) and carboxyethylated (CEL-modified) peptides in tryptic digests of proteins from human plasma, based on the precursor ion approach, earlier established for Amadori compounds [103]. The verification of results and identification of individual glycation sites relied on LIT-Orbitrap-MS analysis. Overall 21CML-modifications sites were identified in 17 proteins including only 2 sites K_{88} and K_{396} in HSA [105]. The same procedure were applied to characterize tryptic peptides (and corresponding glycation sites) with AGE-modified arginine residues [106]. It was shown that 42 plasma proteins are modified by their arginine residues with Glarg, glyoxal-derived dihydroxyimidazolidine (GD-HI), MG-H and methylglyoxal-derived dihydroxyimidazolidine (MGD-HI) [106]. In both strategies [105, 106] were no step of enrichment of AGE-modified peptides which simplifies the analysis and improves the robustness. However, the products can be reliably separated in longer LC gradients, whereas AGE-modified sites can be assigned not only by characteristic mass increments, but also by characteristic fragmentation patterns of in vitro glycated model peptides [24, 107, 108].

Different MS-based methods were developed for characterization of individual glycation sites in plasma proteins.

3. Label-free relative quantification of glycation occupancy at individual protein sites

Label-free relative quantification (LFQ) is the widely used method for biomarker discovery. It is based on the relative comparisons of the abundances (expressed as peak areas, heights or spectral counts) of individual analytes in control and experimental samples [109].

The first insight in the potential biomarker value of glycated proteolytic peptides was provided by the Hoffmann's group in 2010 [110]. In their first pilot study the early glycation patterns in HSA in blood samples obtained from five T2DM patients was addressed. The experimental procedure included protein concentration determination, two steps of trypsin digestion, BAC, filtration on Centricon YM-10 cartridges (to remove high molecular mass cleavage products and aggregates), HPLC separation on C18 trap column and C18 nano-column coupled with electrospray ionization - quadrupole-quadrupole-time-of-flight MS (ESI-QqTOF). The MS analysis was performed in the information-dependent acquisition (IDA) mode with CID for fragmentation. Tandem mass spectra were automatically processed with MASCOT (Matrix Science Ltd) against the SwissProt database and also confirmed by manual interpretation [110]. In most fragment ion spectra, the ions of glycated peptides showed intense signals corresponding to consecutive neutral losses of 18 (–H2O), 36 (−2× H2O), 54 (−3× H2O, pyrylium ion) and 84 (−3× H2O–HCHO, furylium ion) units. These patterns of neutral losses are characteristic for peptides, containing a carbohydrate moiety [99, 105]. Quantification relied on integration of specific extracted ion chromatograms (XICs, $m/z \pm 0.02$) at characteristic retention times (t_R). The BUP approach revealed 18 fructosamine-modified peptides identified by their fragmentation patterns in the plasma samples. Relative quantification showed that 15 glycated peptides were detected with quite similar intensities of corresponding signals in all T2DM samples, whereas two glycation sites showed dramatically different abundances, which could indicate individual, maybe disease-specific, alteration of glycation patterns [110].

To understand the differences in the levels of site-specific Amadori modifications, observed between healthy individuals and T2DM patients, five blood samples

from poor glycemic control (HbA1c ≥ 6.5%) and four non-diabetic participants were used for BUP experiment [83]. The procedure of sample preparation was modified. Filtration step was replaced with SPE on C18-gel loader StageTips, whereas LC–MS analysis followed the procedure of Frolov and Hoffmann [110]. This strategy revealed 52 glycated peptides in T2DM plasma representing 47 glycated lysine residues in12 proteins (HSA, Ig kappa and lambda chain C region, fibrinogen (alpha, beta and gamma chains), complement C3,alpha-2-macroglobulin, serotransferrin, apolipoprotein A-I, and haptoglobin). The Mann–Whitney U-test allowed splitting these peptides into three groups based on the difference of integrated peak area. Five peptides were detected only in T2DM plasma and represented the first group – T2DM-specific sites. The second group included 15 peptides detectable in T2DM plasma at significantly higher levels than in control plasma samples. And third group represented 32 peptides detected inT2DM and control plasma samples at similar intensities, i.e. did not exhibit biomarker properties. It is necessary to take into account that the prevalence of not affected sites could be explained by small size of the cohorts, which could be insufficient for reliable conclusions [83].

Therefore, recently, we extended this approach to larger cohort size and established an integrated biomarker, based on multiple glycation sites [84]. This experiment employed T2DM female patients (n = 20 with the serum levels of HbA1c ≥ 7.5%) and age-matched normoglycemic women (n = 18 with the levels of HbA1c ≤ 6.5%). After nanoLC–MS by the above described workflow, all peptide signals were matched to the most complete glycation site database from Zhang et al. [87, 102] and results of our previous work [83] (in total more than 350 sites in plasma proteins). This approach resulted in identification of 51 Amadori peptides, 42 of which were differentially abundant in diabetic and normoglycemic controls. These peptides represented in total nine plasma proteins (HSA Ig kappa chain C region, complement C4-A, alpha-2-macroglobulin, serotransferrin, apolipoprotein A-I, ceruloplasmin precursor, Vitamin D-binding protein precursor and FLJ00385 protein), with half-lives from 2 to 21 days. Based on these differentially modified sites, we proposed an integrated biomarker based on multiple protein-specific Amadori peptides. The validation of this biomarker relied on linear discriminant analysis (LDA) with random sub-sampling of the training set and leave-one-out cross-validation (LOOCV), which resulted in an accuracy, specificity, and sensitivity of 92%, 100%, and 85%, respectively. In this context, it is logical to assume that a biomarker strategy, based on multiple specific glycation sites in plasma proteins, could essentially increase the efficiency of glycemic control and disease prediction.

Due to a high heterogeneity of AGE structures and relatively low abundances of individual AGEs at specific amino acid residues, label-free analysis of modification sites in advanced glycated proteins is rather challenging [105]. Recently, we reported plasma patterns of amide AGEs in the patients, featured with different obesity status and degree of glycaemic control, i.e. we compared four cohorts represented with hyperglycaemic and normoglycemic lean and obese individuals [111]. Although sample preparation followed our well-established pipeline [105, 106], at the stage of LC–MS analysis we employed gas phase fractionation (18 *m/z* intervals in the overall mass range 100–1400 *m/z*), that allowed higher discovery rates of AGE-modified peptides. As a result, altogether 15 advanced glycated sites in 11 proteins were detected in plasma of hyperglycaemic patients. Thereby, the relative contents of two sites, representing acetylation at K_{199} in HSA ($LK_{acetyl}CASLQK$) and formylation at K_{51} in apolipoprotein A-II ($SK_{formyl}EQLTPLIK$) were significantly ($p < 0.05$) higher in patients with poor glycemic control [111]. Thus, the peptides, representing the sites, can be considered as potential marker of hyperglycemia. The follow-up study, involving larger cohorts and addressing a wider array of

AGEs [112] identified 36 sites in 22 highly abundant proteins in individual plasma samples obtained from T2DM patients with long-term disease. Major modifications were Glarg (11 modification sites), CMA (5), N^{ϵ}-(formyl)lysine (8), N^{ϵ}-(acetyl) lysine (7), and CML (7). No significant changes were observed between control and T2DM group [112].

Brede and co-authors [101] established fast and high-throughput analysis of several glycated peptides of HSA. The trypsin digestion was done in 76% acetonitrile. Thereby, the authors skipped BAC enrichment and pre-cleaning with SPE. Before qualitative LC–MS/MS analysis, acetonitrile was evaporated from the samples and tryptic peptides were loaded on a C18 reversed phase trap column and separated on an analytical column coupled on-line to a QqTOF mass spectrometer. Quantitative LC–MS/MS analysis was performed by separation on BEHC18 column coupled with a Xevo TQ-S triple quadrupole tandem mass spectrometer operated in multiple reaction monitoring (MRM) mode. This method allows identification of only several glycated peptides from high abundant plasma protein HSA with the modification sites K_{525}, K_{137}, K_{12}, and K_{414}, respectively. Glycated peptide contained K_{525} was used in the quantitative analysis. The level of glycation at K_{525} was strongly correlated with HbA1c (r = 0.84) for patients without ESRD. In theT2DM patients with ESRD had a higher ratio of K_{525}/HbA1c on average, provides an excellent incentive for exploring the method as a supplement to HbA1c for detecting increased blood glucose in these patients [101].

In the work of Rathore and co-authors [90], both AGE-modified and Amadori-modified peptides were used for prediction of pre-diabetes in an integrated biomarker approach. Thereby, the authors focused on glycation of the major plasma protein - HSA. Based on HbA1c levels, the patients were categorized as healthy (n = 20) and pre-diabetic (n = 20). The digestion strategy relied on RapiGest – a detergent, which could be removed from the samples by precipitation with strong acids upon digestion and pre-cleaning on C18 zip-tip columns. Tryptic hydrolysates were separated on C18-reverse phase column coupled to Q-Exactive Orbitrap MS operated in parallel reaction monitoring (PRM) mode based on the information about precursor *m/z*, and charge state obtained during DDA (targeted label-fee approach). Normalized peak areas of glycated peptides were used for a two-tailed, unpaired, non-parametric t-test and two way ANOVA to determine the significance of glycation. As consequence, four CML- or Amadori modified peptides corresponding to 3 glucose sensitive lysine residues K_{36}, K_{438}, and K_{549}, respectively showed significantly higher abundance in pre-diabetes than control. Additionally, the abundance of three of these peptides (K_{Am}QTALVELVK, K_{CML}VPQVSTPTLVEVSR and FK_{CML}DLGEENFK) was >1.8-fold in pre-diabetes, which was significantly higher than the differences observed for fasting blood glucose (FBG), 2 h postprandial glucose (PPG), and HbA1c. Further, the four glycated peptides showed a significant correlation with FBG, PPG, HbA1c, triglycerides, very low density lipoproteins (VLDL), and high-density lipoproteins (HDL). It indicates that glycated peptides, containing glucose-sensitive lysine residues K_{36}, K_{438} and K_{549} of HSA could be potentially useful markers for prediction of pre-diabetes [90].

As can be seen from the overview, LFQ provides an essential advantage in quantification of relative glycation rates at practically all available modification sites in multiple proteins. Therefore, this approach gives a direct access to combining multiple biomarkers by simultaneous consideration of several proteins with different half-life times. This allows monitoring any long- and short-term fluctuations of blood glucose concentrations, as for any desired duration of the observation period a protein with appropriate half-life can be found.

Currently, accumulation of the information on prospective biomarker sites is necessary. In further studies, this information needs to be verified in large cohorts to

assess the predictive potential of these markers. However, on this way, the limitations of label-free approach become critical. Indeed, LFQ is disadvantages for analysis of large cohorts due to sensitivity of electrospray ionization technique (ESI) to multiple factors, which is manifested as matrix effects [113]. Thus, the analysis conditions (e.g. temperature, experimenter, column condition) must be as constant as possible, that is difficult to achieve with big batch sizes. And duration of batch analysis can be rather long, as prolonged gradients are often used to improve peptide separation [114]. To overcome these limitations, absolute quantification strategies can be employed [86].

4. Absolute quantification of prospective biomarkers in blood plasma with isotopically labeled internal standards

Absolute quantification of glycation rates at individual modification sites by means of isotopically labeled standards was for the first time proposed by Zhang and co-workers in 2013 [86]. For the early diagnosis of T2DM glycation at the most abundant human plasma protein HSA was monitored by quantitative analysis of its characteristic tryptic peptides. Thereby, all probands were classified into three groups: T2DM (n = 73), IGT (n = 63), and NGT (n = 253). In this study ^{18}O-labeling was used to screen glucose-sensitive and glucose-insensitive peptides within HSA-derived peptides. Glucose-sensitive peptides tested as biomarker candidates for T2DM in a clinical plasma samples, and glucose-insensitive peptide was selected as the internal standards. Three peptides (LDELRDEGK (K_{190}), FKDLGEENFK (K_{12}), and KVPQVSTPTLVEVSR (K_{414})) showed significant different in their concentrations in the T2DM group compared with the IGT group. Among them two peptides FKDLGEENFK and KVPQVSTPTLVEVSR exhibited significant differences between both NGT/IGTand IGT/T2DM groups indicating that these peptides could be used as potential biomarkers for the early diagnosis of T2DM. It is important to mention that peptides FKDLGEENFK and KVPQVSTPTLVEVSR showed excellent sensitivities (97.23 and 94.47%, respectively) and specificities (93.65 and 98.41%, respectively) between the NGT/IGT groups. For the NGT/IGT and T2DM groups sensitivity of peptides FKDLGEENFK and KVPQVSTPTLVEVSR was 97.06 and 99.27%, respectively, and specificities was 97.23 and 94.47%. It indicates that these peptides could be prospective biomarkers for the early diagnosis of T2DM [86].

The internal standardization with stable isotope-labeled synthetic peptides and related stable isotope dilution techniques are widely used in glycation research [84, 95, 111, 112]. A stable isotope label can be easily introduced in the step of amino acid building block during the synthesis of Amadori-modified peptides [115]. These peptides can be directly used in the stable isotope dilution approach for absolute quantification (AQUA) [116]. Implementation of synthetic Amadori-modified peptides increases robustness, precision and accuracy of biomarkers analysis [117].

In the classical way this strategy was established for glycated peptides by Spiller and co-workers [118], who applied six standard synthetic peptides containing two isotope-labeled residues (introduced by Fmoc-($^{13}C_6$,^{15}N) leucine and Fmoc-($^{13}C_5$,^{15}N) proline) along with Amadori modification, introduced by the global post-synthetic glycation approach [119]. The peptides represented six glycation sites in HSA – TCVADESAENCDKSLHTLFGDK (K_{64}), SLHTLFGDKLCTVATLR (K_{73}), AACLLPKLDELRDEGK (K_{181}), ADLAKYICENQDSISSK (K_{262}), VFDEFKPLVEEPQNLIK (K_{378}) and KLVAASQAALGL (K_{574}). The standard mixtures were spiked (25 μL, 1.2 μmol/L) to the tryptic digest, obtained from five T2DM patients and five non-diabetic individuals and, after BAC enrichment and SPE, analyzed by a hybrid quadrupole-linear ion trap MS in the MRM mode with three specific Q1/Q3 *m/z* ranges (transitions) for each analyte [118].

All six analytes and corresponding standard peptides could be detected in all ten plasma samples. The quantities varied from 22.6 ± 3.3 to 180.3 ± 8.8 pmol per mg plasma protein. Also it was shown that content of all six glycated peptides were statistically different between the two cohorts. This study demonstrated the applicability of the AQUA technique to quantification of glycation sites in plasma proteins. In agreement with the results of LFQ studies [83], individual HSA glycation sites responded to hyperglycaemia in different ways. Interestingly, significantly higher, in comparison to others, abundance of glycated sites K_{64} and K_{378} was observed [118].

For absolute quantification of glycated peptides by the standard isotope dilution technique Prof. Stefanowicz and co-workers used bi-labeled peptides that contained stable isotope label, introduced as $^{3}C_6{}^{15}N_2$-lysine, and comprise a dabsyl moiety that cleaves during digestion procedure [115]. Later on we have shown applicability of these bi-labeled glycated peptides for the absolute quantification of individual glycation sites in plasma proteins [120]. Based on the previous label-free quantification [83] the prevalent glycated peptides with biomarker properties were chosen. The Amadori-modified standard peptides DSTYSLSSTLTLSK$_{Am}$ADYE$^{13}C_6{}^{15}N_2$-KK$_{Dab}$K represented the sequence of Ig kappa chain C region protein, ADLAK$_{Am}$YICENQDSISS$^{13}C_6{}^{15}N_2$-KK$_{Dab}$K and VFDEFK$_{Am}$PLVEEPQNLI$^{13}C_6{}^{15}N_2$-KK$_{Dab}$K corresponded to the HSA sequence were synthesized. The mixture of standard stable isotope-labeled peptides (20 pmol each) was added to aliquots of plasma samples (of 5 T2DM patients and 5 non-diabetic individuals). After tryptic digestion, samples were analyzed by QqTOF-MS. Glycated peptides from plasma samples and standards were annotated in MS scans (*m/z* 400–2000) by their t_R, *m/z* and isotopic patterns and confirmed by MS/MS analysis using Orbitrap Elite MS. The corresponding MS profiles clearly represented isotopic patterns of both light and heavy peptides with good sensitivities that gave access to reliable quantification by peak areas of analytes and corresponding internal standards. All three potential biomarker peptides demonstrated a significantly higher (in 1.5–1.9-fold) content in diabetic patients, in comparison to that in non-diabetic controls. The obtained results were cross-validated by label-free quantification performed in an independent RP-HPLC-ESI-QqTOF-MS study [120]. The resulting fold changes were close to those observed with the stable isotope dilution approach, proposed by Spiller et al. [118]. It indicates similar power of the both methods for absolute quantification of individual glycation sites in blood plasma proteins [120].

Later Spiller *et al.* [97] studied the glycation degrees of 27 glycation sites representing nine plasma proteins in 48 newly diagnosed male T2DM patients and 48 non-diabetic individuals. After protein digestion with trypsin, samples were spiked with concentration-balanced mixture of synthetic ^{13}C,^{15}N-labeled glycated peptides (synthesized according Spiller at al [118]) as internal standards. The quantification was based on MRM using specific transitions for each targeted peptide and isotope-labeled internal peptide standards. The samples from two groups of participants were evaluated by different statistical tests (Kolmogorow-Smirnow, Mann–Whitney, and *t* test), classified by a decision tree algorithm using HbA1c in combination with each glycated peptide. Also to find the best feature set for classification, support vector machine-recursive feature elimination (SVM-RFE) method was performed for all glycated peptides and clinical parameters of participants, including HbA1c, fasting plasma glucose (FPG), body mass index (BMI), etc. The most interesting results were obtained for glycated peptide AVGDKLPECEAVCGKPK (K_{141}) from haptoglobin which half-life time is 2–4 days. The combination of two biomarkers of T2DM glycated K_{141} of haptoglobin and HbA_{1c}provided a sensitivity of 94%, a specificity of 98%, and an accuracy of 96% to identify T2DM.But a set of 15 features considering three glycation sites in HSA, K_{141} in haptoglobin, and 11 routine laboratory measures of T2DM, metabolic syndrome, obesity, inflammation, and insulin

resistance provided a sensitivity of 98%, a specificity of 100%, and an accuracy of 99% for newly diagnosed T2DM patients. This study shows great potential of glycation sites in plasma proteins providing an additional diagnostic tool for T2DM and elucidating that the combination of these sites with HbA1c and FPG could improve the diagnosis of T2DM. The combination of both biomarkers HbA1c and glycated haptoglobin with half-life times 2 to 4 days is sensitive to long- and short-term fluctuations of blood glucose concentrations [97].

Selected 27 glycated peptides were tested further in the quality of multiple biomarker set [121]. For this plasma samples from 48 patients with duration of T2DM for more than 10 years, 48 non-diabetic individuals and 20 pre-diabetic persons we examined. The strategy of analysis was the same as described above [97]. In long-term controlled T2DM patients, 27 glycated peptides were detected at significantly higher levels and provided moderate diagnostic accuracies (ACCs) from 61 to 79%, resulting in sub-grouping of patients in three distinct clusters. In this study, a feature set of one glycated peptides from haptoglobin (K_{141}) and 6 established clinical parameters provided an ACC of 95%. The same number of clusters was identified in pre-diabetic males (ACC of 95%) using a set of 8 glycation sites (mostly from HSA). Re-examination of all patients present in one cluster showed progression of pre-diabetic state or advanced towards diabetes in the following five years. Overall, the studied glycation sites can play a role of promising biomarkers for sub-grouping pre-diabetic patients to estimate their risk for the development of T2DM [121].

Together with our recent report [84] these work clearly indicate under-explored potential of integrated peptide biomarkers. Moreover, it is well seen, that absolute quantification approaches are preferable, due to their higher precision and lower method-related dispersion. Thus, it is obvious, that after the explorative LFQ-based study, follow-up absolute quantification screening of large cohorts is necessary.

Besides ^{18}O-labeling and AQUA, tag-based labeling approaches were used for quantification of glycation sites. Thus, Qiu*et al*. proposed the use of isobaric tags for relative and absolute quantification (iTRAQ) to reveal differences in HSA glycation patterns between healthy individuals and diabetic patients [88]. The authors described *in vitro* and *in vivo* experiments have been carried out to evaluate the impact of HSA glycation on the binding to anticoagulant drugs (warfarin and heparin). Plasma samples from 32 diabetic patients and 33 healthy individuals were treated with polyethylene glycol for precipitation of immunoglobulins. After this, the glycated albumin (GA) was separated using BAC. Trypsin digested GA was separated on C18-AQ analytical column and analyzed using a LTQ-Orbitrap Velos Pro MS operated in DDA mode with CID and further with ETD fragmentation. For quantitative analysis, the digested samples were labeled with iTRAQ 8-plex reagents. After labeling, the 8 samples with different mass tags were evenly mixed and glycated peptides with iTRAQ labels were extracted from the pooled samples using BAC. MS analysis was performed on a LTQ-Orbitrap Fusion MS operated in the DDA mode for CID-MS/MS and HCD-MS^3. A total of 49 glycation sites (including 43 glycated lysines and six glycated arginines) on GA were successfully identified using this approach. Among them seven glycation sites, R_{81}, R_{117}, R_{186}, R_{257}, K_{313}, R_{410} and K_{541}, were discovered for the first time. It is interesting that glycation at sites K_4, R_{81}, R_{117}, K_{439}, K_{519}, K_{538}, K_{541}, K_{557} and K_{573} were specifically present in diabetic patients, while two sites, R_{410} and K_{436}, were found only in healthy subjects. Altogether 21 glycation sites were quantified, and 19 of them, including K_{51}, K_{64}, K_{93}, K_{162}, K_{199}, K_{233}, K_{262}, K_{313}, K_{323}, K_{378}, K_{402}, K_{414}, K_{466}, K_{475}, K_{525}, K_{545}, K_{557}, K_{564} and K_{574}, showed statistically enhanced glycation during diabetes [88].

Examples of methods used for absolute quantification of prospective biomarkers in blood plasma with isotopically labeled internal standards are summarized in **Table 2**.

#	Proteins and sites of glycation	Peptide labeling	MS	Method power	Ref
1	HSA (K_{414})	^{18}O-labeling	ESI-QqTOF	Sens. 99.27%, spec. 94.47%	[86]
2	HSA (K_{64}, K_{73}, K_{181}, K_{262}, K_{378}, K_{574}).	*L*-leucine-$^{13}C_6$,^{15}N, *L*-proline $^{13}C_5$,^{15}N	ESI-QqLIT	Statistical difference between control and T2DM: $p < 0.0001$ or $p < 0.05$	[118]
3	Ig kappa chain C region protein (K_{75}), HSA (K_{286}, K_{402})	*L*-lysine-$^{3}C_6{}^{15}N_2$ and dabsyl moiety	ESI-QqTOF	Significantly higher (in 1.5–1.9-fold) content in T2DM	[120]
4	Haptoglobin (K_{141}), HSA (K_{93}, K_{262}, and K_{414})	^{13}C,^{15}N-labelling	ESI-QqLIT	Sens. 98%, spec. 100%, accur. 99%	[97]
5	Haptoglobin (K_{141}), HSA (K_{262}, K_{378}, K_{73}, K_{525}, K_{574}, K_{359}, K_{174}, K_{64}), serotransferrin (K_{683})	^{13}C,^{15}N-labelling	ESI-QqLIT	Diagnostic accuracy 95%	[121]
6	HSA (K_{51}, K_{64}, K_{93}, K_{162}, K_{199}, K_{233}, K_{262}, K_{313}, K_{323}, K_{378}, K_{402},K_{414}, K_{466}, K_{475}, K_{525}, K_{545}, K_{557}, K_{564} and K_{574})	^{18}O-labeling	ESI-LTQ-Orbitrap	Statistical difference between control and T2DM $p < 0.05$	[88]

Table 2.
Overview of methods used for absolute quantification of prospective biomarkers in blood plasma with isotopically labeled internal standards. Accuracy (accur.), sensitivity (sens.), specificity (spec.)

5. Conclusions

T2DM is one of the most widely spread metabolic disorders and most often discovered at the step of complications, which makes therapy less efficient and more expensive. HbA1c provides information about changes in glycaemic status over three months, and, hence, is insensitive to short-term glucose fluctuations preceding the disease. In this context, using individual glycation sites as T2DM biomarkers might provide a good solution of this problem. Precise and reliable quantification of glycated peptides is a prerequisite for establishing biomarkers and developing clinical diagnostics of T2DM. Different methods are established and qualified for the quantification of individual glycation sites in plasma proteins using label-free or absolute quantification. Due high precision it is applicable to use in the combination with HbA1c for screening of large patient cohorts for early diagnosis of T2DM, therapy control, sub-typing disease stages, and the prognosis of complication risks. Obviously, the biomarker potential of glycated peptides is still mostly unknown. On one hand, more explorative studies are necessary to discover new biomarker candidates. On the other – these biomarker candidates need to be confirmed in wide-scale screening in large cohorts with reliable absolute quantification methods.

Acknowledgements

The reported study was funded by Russian Foundation for Basic Research according to the research project #18-34-00927 and Deutsche Forschungsgemeinschaft (DFG, project # FR3117/2-3).

Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

2-DE	Two-dimensional gel electrophoresis
3DG-H1	3-deoxyglucosone-derived hydroimidazolone 1
ACC	diagnostic accuracy
ACN	acetonitrile
AGEs	advanced glycation end products
AP-1	activator protein-1
BAC	boronate affinity chromatography
BEH	ethylene bridged hybrid
BMI	body mass index
BUP	bottom-up proteomic
CEA	N^{δ}-(carboxyethyl)arginine
CEL	N^{ε}-carboxyethyl lysine
CID	collision induced dissociation
CMA	N^{ω}-carboxymethylarginine
CML	N^{ε}-carboxymethyl lysine
DDA	data dependent acquisition
DIA	data-independent acquisition
DOLD	3-deoxyglucosonederived lysine dimer
ELBIA	enzyme-linked boronate immunoassay

ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
ESI-QqLIT	electrospray ionization - quadrupole-linear ion trap
ESI-QqTOF	electrospray ionization - quadrupole-quadrupole-time-of-flight MS
ESRD	end-stage renal disease
ETD	electron-transfer dissociation
FBG	fasting blood glucose
FPG	fasting plasma glucose
G-H1	glyoxal-derived hydroimidazolone 1
GA	glycated albumin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC–MS	gas chromatography coupled with mass spectrometry
GD-HI	dihydroxyimidazolidine
Glarg	glyoxal-derived hydro-imidazolinone
GODIC	glyoxal derived imidazolium crosslinking
GOLD	glyoxal-derived lysine dimer
HbA_{1c}	glycated hemoglobin
HCD	higher-energy collisional dissociation
HDL	high-density lipoproteins
HPLC	high-performance liquid chromatography
HPLC–MS	high-performance liquid chromatography coupled with mass spectrometry
ID	internal diameter
IDA	information-dependent acquisition
Ig	immunoglobulin
IGT	impaired glucose tolerance
iTRAQ	isobaric tags for relative and absolute quantification
LC-IT-TOF	liquid chromatography – ion trap – time-of-flight
LC–MS/MS	liquid chromatography coupled with tandem mass spectrometry
LDA	linear discriminant analysis
LFQ	label-free relative quantification
LFIA	lateral flow immunoassay
LIT	linear ion trap
LOD	limit of detection
LOOCV	leave-one-out cross-validation
LOQ	limit of quantification
MG-H1	methylglyoxal-derived hydroimidazolone 1
MGD-HI	methylglyoxal-derived dihydroxyimidazolidine
MOLD	methylglyoxal-lysine dimer
MRM	multiple reaction monitoring
MWCO	molecular weight cut off
MS	mass spectrometer
MS/MS	tandem mass spectrometry
NBT	nitroblue tetrazolium
NF-kB	nuclear factor k-light-chain-enhancer of activated B cells
NGT	normal glucose tolerance
PPG	2 h postprandial glucose
PRM	parallel reaction monitoring
SCX	strong cation exchange
SDS	sodium dodecyl sulfate
SVM-RFE	support vector machine-recursive feature elimination
STAT	signal transducer and activator of transcription

T2DM	type 2 diabetes mellitus
UHPLC	ultra-high-pressure liquid chromatography
UV	ultraviolet detector
VLDL	very low density lipoproteins
XICs	extracted ion chromatograms

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