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Protein glycation – biomarkers of metabolic dysfunction and early-stage decline in health in the era of precision medicine

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ABSTRACT

Protein glycation provides a biomarker in widespread clinical use, glycated hemoglobin HbA_{1c} (A1C). It is a biomarker for diagnosis of diabetes and prediabetes and of medium-term glycemic control in patients with established diabetes. A1C is an early-stage glycation adduct of hemoglobin with glucose; a fructosamine derivative. Glucose is an amino group-directed glycating agent, modifying N-terminal and lysine sidechain amino groups. A similar fructosamine derivative of serum albumin, glycated albumin (GA), finds use as a biomarker of glycemic control, particularly where there is interference in use of A1C. Later stage adducts, advanced glycation endproducts (AGEs), are formed by the degradation of fructosamines and by the reaction of reactive dicarbonyl metabolites, such as methylglyoxal. Dicarbonyls are arginine-directed glycating agents forming mainly hydroimidazolone AGEs. Glucosepane and pentosidine, an intense fluorophore, are AGE covalent crosslinks. Cellular proteolysis of glycated proteins forms glycated amino acids, which are released into plasma and excreted in urine. Development of diagnostic algorithms by artificial intelligence machine learning is enhancing the applications of glycation biomarkers. Investigational glycation biomarkers are in development for: (i) healthy aging; (ii) risk prediction of vascular complications of diabetes; (iii) diagnosis of autism; and (iv) diagnosis and classification of early-stage arthritis. Protein glycation biomarkers are influenced by heritability, aging, decline in metabolic, vascular, renal and skeletal health, and other factors. They are applicable to populations of differing ethnicities, bridging the gap between genotype and phenotype. They are thereby likely to find continued and expanding clinical use, including in the current era of developing precision medicine, reporting on multiple pathogenic processes and supporting a precision medicine approach.

1. Introduction – protein glycation in the clinical setting

Protein glycation is the spontaneous, non-enzymatic reaction of proteins with simple reducing sugars and related metabolites. In the clinical setting, the major glycating agent is glucose which reacts with amino groups of N-terminal and lysine residues of proteins to form an initial Schiff`s base, with following rearrangement to form N-(1-deoxy-D-fructos-1-yl)amino acids or fructosamines [1]. For lysine residues, the adduct is N_{ε}-1-deoxyfructosyl-lysine (FL), with analytical surrogate, furosine [2] (Fig. 1a). Glycation of proteins by glucose to the fructosamine stage is classified as early glycation. In later stage reactions, fructosamine degrades to stable endstage adducts called advanced glycation endproducts (AGEs). Other important glycating agents are reactive α-oxoaldehyde metabolites, particularly methylglyoxal (MG), glyoxal and 3-deoxyglucosone. Dicarbonyls from mainly arginine-derived hydroimidazolone AGEs as the dominant product, typically accounting for >90% total glycation adducts formed [3,4]. Other minor glycation adducts include N_{ε}-carboxymethyl-lysine (CML) and N□-carboxymethyl-arginine (CMA) formed from glyoxal, and N_{ε}-carboxyethyl-lysine (CEL) from MG. Glucose and MG are precursors of major quantitative early-stage glycation adducts and AGEs, FL and hydroimidazolone MG-H1, respectively. The formation of AGEs, CML from FL and pentosidine from pentose precursors, involves oxidation and these processes are called glycoxidation (Fig. 1b). Glycation adducts are present as two major forms: glycation adduct residues of proteins –

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Abbreviations

Ab	antibody
A1C	glycated hemoglobin HbA_{1c}
ACE	angiotensin converting enzyme
AGE	advanced glycation endproduct
ARB	angiotensin receptor blocker
ASD	autism spectrum disorder
BMI	body mass index
CAC	coronary artery calcification
CCP	citrullinated cyclic peptide
CEL	N_{ε}-carboxyethyl-lysine
CIMT	carotid intima-media thickness
CKD	chronic kidney disease
CMA	$N_{\square}$-carboxymethyl-arginine
CML	N_{ε}-carboxymethyl-lysine
CVD	cardiovascular disease
DCCT	Diabetes Control and Complications Trial
3DG-H	3-deoxyglucosone-derived hydroimidazolone
DT	dityrosine
EDRF	early decline in renal function
ELOVL2	elongation of very long-chain fatty acids protein-2
FADS	polymorphism of fatty acid desaturase
FL	N_{ε}-1-deoxyfructosyl-lysine
FN3K	fructosamine 3-kinase
G6PD	glucose-6-phosphate dehydrogenase
GA	glycated albumin
GCKR	glucokinase regulator
GFR	glomerular filtration rate
GG	glycosylation gap
G-H1	glyoxal-derived hydroimidazolone
Glo1	glyoxalase 1
GSP	glucosepane
GWAS	genome-wide association studies
HDL	high density lipoprotein
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LDL	low density lipoprotein
LR	likelihood ratio
MetSO	methionine sulfoxide
MG	methylglyoxal
MG-H1	methylglyoxal-derived hydroimidazolone
NBT	nitroblue tetrazolium
NFK	N-formylkynurenine
3-NT	3-nitrotyrosine
OA	osteoarthritis
PAD	peripheral artery disease
PTC	renal proximal tubular epithelial cells
SNP	single nucleotide polymorphism
RA	rheumatoid arthritis
ROS	reactive oxygen species
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TER	transcapillary escape rate

also called “protein-bound glycation adducts” and glycated amino acids or glycation free adducts. Cellular proteolysis forms glycation free adducts which are released into plasma and excreted in urine. There is also absorption of glycation free adducts and small glycated peptides from digestion of glycated proteins in food [5]. Processes of formation and metabolic transit of protein glycation are summarised schematically (Fig. 1c). Protein glycation occurs in the cellular and extracellular compartments. Glycated proteins are also ingested and those degraded to small glycated peptides and free adducts are absorbed in the portal vein. Albumin cycles from plasma into interstitial fluid, lymph and returns to plasma – with some leakage through renal glomeruli and return to venous circulation by the renal albumin retrieval pathway of proximal tubular epithelial cells (PTCs) [6]. The major route of excretion of glycation adducts is as free adducts in urine, with minor excretion in urinary albumin, other proteins and peptides. Glycation free adducts with molecular mass <500 Da pass readily through the glomerular filter into glomerular filtrate and are reabsorbed and secreted in the renal proximal tubules, likely by amino acid transporters, producing usually high and characteristic fractional excretions [7,8].

In this review, we describe the clinical diagnostic application of glycation adducts as biomarkers. Glycation adducts may be biomarkers used for diagnosis, risk of developing a health disorder or disease and monitoring of the response to treatment. The steady-state levels of glycation adduct residues of proteins reflect the rates of formation, degradation and repair of the glycated protein in the compartments where the protein substrate is located [9]. Glycation adducts provide reports on different aspects of metabolism [9] (Table 1). The levels of glycation free adducts in plasma reflects the balance between fluxes of free adducts released into the vasculature from tissues (including absorption from digested food in the portal vein) and renal clearance [10], increasing profoundly in experimental nephrectomy and clinical renal failure [7,11]. The urinary flux of glycation free adducts reflects the total body flux of formation of glycation adducts with a contribution from glycation free adducts absorbed from food [7,11,12]. Factors affecting the level of glycated protein biomarker are: concentration of the protein substrate, concentration of the glycating agent and duration of exposure to it, and turnover and repair and replacement of the glycated protein [5]. Other non-glycation based physiological factors provide interferences in biomarker reporting of physiological glycation [13]. Robust measurement of glycation adducts in the clinical chemistry laboratory has been challenging which recent advances in application of stable isotopic dilution analysis LC-MS/MS have overcome [9,14]. The strength and weakness of different analytical methods and overview of steps in development and validation for clinical use are summarised in Table 2. With optimized methodologies and protocols now available, protein glycation provides unique and valuable clinical and investigational biomarkers.

2. Glycated hemoglobin A1C

Glycated hemoglobin, HbA_{1c} or A1C, is widely used for the diagnosis and monitoring of glycemic control of diabetes, in both patients with type 1 diabetes mellitus (T1DM) and patients with type 2 diabetes mellitus (T2DM). Current guidelines advise A1C is measured at least biannually in patients with diabetes [15]. A1C is also used for the diagnosis of prediabetes – a condition that precedes development of T2DM [16]. It is formed by the reaction of glucose with unglycated hemoglobin, HbA_0. The major adduct formed is the fructosamine derivative of the N-terminal valine residue of the beta-chain, ßval-1, with also glucose modification at αlys-61 and βlys-66 [17–19]. There are other minor forms of glycated hemoglobin resolved from A1C in conventional cation exchange chromatography analysis used in clinical chemistry, representing <1% total Hb: HbA_{1a1}, HbA_{1a2} and HbA_{1b}, which are ßval-1 adducts of fructose-1,6-bisphosphate, glucose-6-phosphate and pyruvate, respectively, and HbA_{1d} (aldimine Schiff’s base) and HbA_{1e} (fructosamine adduct of αval-1) [20–22]. The steady-state level of A1C *in vivo* is influenced by deglycation of glycation adducts by fructosamine 3-kinase (FN3K) [23,24], red blood cell lifespan (mean 120 days) and glycemic control over the previous 90–120 days [25]. A1C is a weighted measure of mean blood glucose concentration during

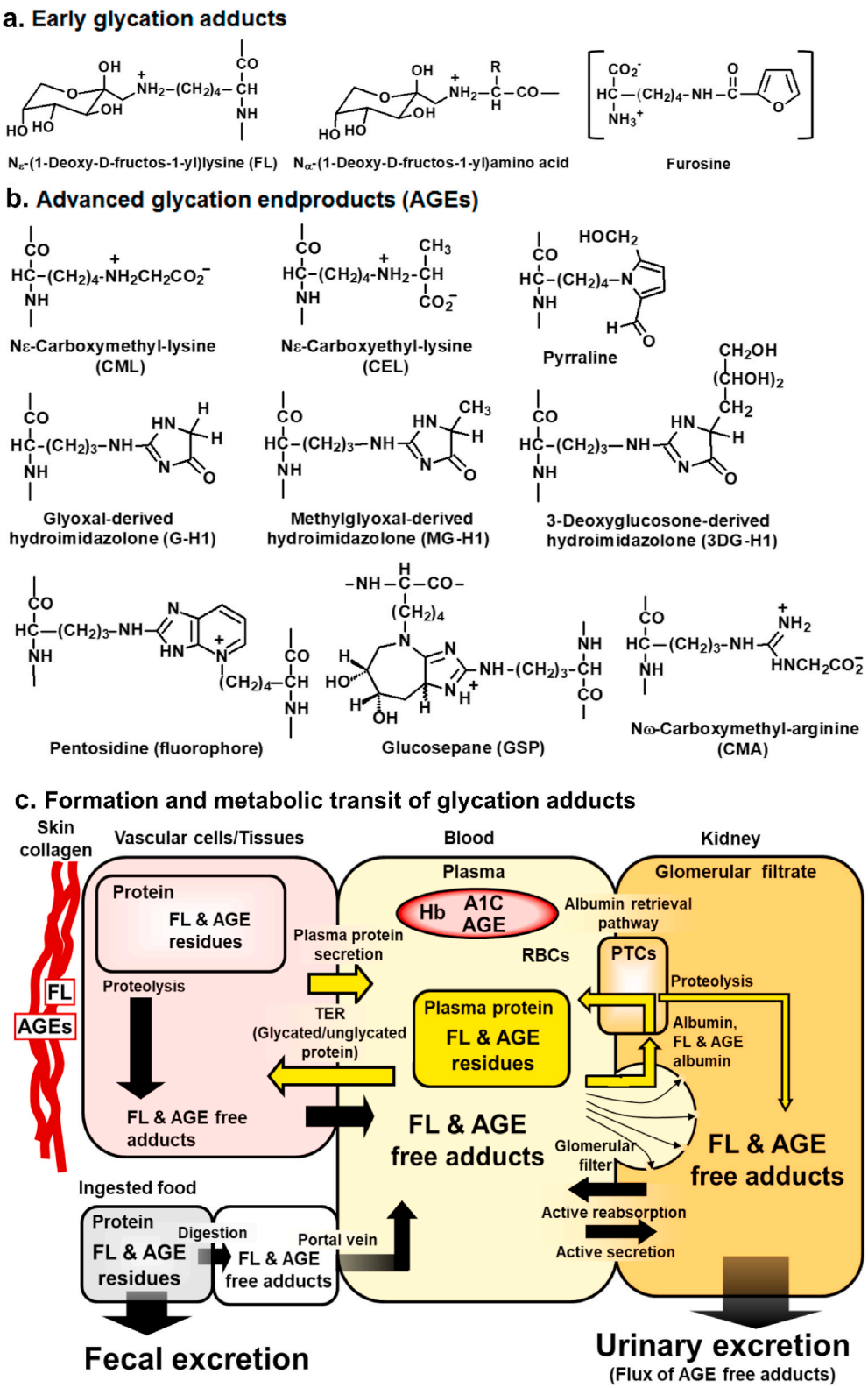

Fig. 1. ***Protein glycation adducts (a. and b.)and formation, physiological processing and metabolic transit of glycated proteins (c.).*** Glycation adducts are shown as adduct residues with peptide bond linkage and ionization of glycation adducts at physiological pH, 7.4. For glycation free adducts, the peptide backbone N- and C-termini are $-NH_3^+$ and $-CO_2^-$, respectively. The square bracket round furosine indicates that it is an analytical surrogate (of FL), formed during acid hydrolysis in pre-analytic processing. Abbreviations: A1C, glycated hemoglobin HbA_{1c}; PTC, proximal tubular epithelial cell; TER, transcapillary escape rate.

the 120 days prior to measurement; blood glucose levels in the 30 days immediately prior to blood sampling contribute approximately 50%, whereas levels in prior 90–120 contribute only 10% [26–28]. A1C is a valuable biomarker of medium-term glycemic control, with the disadvantage that intervention trials to improve dysglycemia in prediabetes and hyperglycemia in diabetes must be at least 3 months duration for responsiveness of A1C [29]. The validity of A1C as a biomarker of glycemic control is impaired when non-glycemic factors influence levels: such as impaired red blood cell synthesis in renal failure [30], unstable hemoglobin variants [31] and dysregulated iron and red blood cell metabolism [32] – including in pregnancy [33]. Good control of hyperglycemia is important in the clinical care of patients with diabetes which, together with control of blood pressure and lipids, decreases the risk of developing debilitating vascular complications of diabetes:

Table 1
Biomarker characteristics of protein glycation adducts.

Glycation adduct	Reporter feature (precursor)	Comment
FL[a]	Glycemic control (glucose)	Major early-stage glycation adduct. Repaired intracellularly by fructosamine 3-phosphokinase [24]. Free adduct absorbed after digestion of food proteins [121].
MG-H1	Dicarbonyl stress (methylglyoxal)	Major AGE. Linked to increased fasting and postprandial glucose exposure, unscheduled glycolysis, insulin resistance and cardiovascular disease [4,12,90,119,122]. Free adduct absorbed after digestion of food proteins [12]. Good stability in samples with delayed processing for epidemiological studies [73]
CML	Glycoxidation glycemic control/(FL and others)	Major AGE. Formed by the oxidative degradation of FL and other sources. CML/FL ratio is an indicator of oxidative stress [123]. Free adduct absorbed after digestion of food proteins [124].
GSP	Glycemic control/glycation crosslinking (FL)	Major glycation crosslink [75]. Major glycation adduct released from joint proteins during early-stage development of osteoarthritis [116]. Good stability in samples with delayed processing for epidemiological studies [73].
Pentosidine	Pentosephosphate pathway activity (pentose metabolite)	Low level pentose sugar-derived glycation crosslink and intense fluorophore. Considered to reflect pentosephosphate pathway activity [125]. Skin collagen pentosidine accounts for 30% variation in SAF [126]
Pyrraline	AGE exposure from food (cooked food)	Glucose-derived AGE formed at high temperatures of culinary processing; originating only from food [127,128]. Urinary pyrraline excretion is linked to amount of AGE absorbed from food [12].

[a] Also applies to N-(1-deoxy-D-fructos-1-yl)amino acids (fructosamine). Abbreviation: SAF, skin autofluorescence.

diabetic nephropathy, diabetic retinopathy, diabetic neuropathy and increased risk of cardiovascular disease (CVD). As a biomarker of glycemic control, A1C is considered a modifiable risk factor for the chronic vascular complications of diabetes. Decrease of A1C by intensive treatment with hypoglycemic drugs is associated with decreased risk of development of diabetic microvascular complications [34–36] and decreased progression of CVD in patients with T1DM [37,38] (Table 3). A1C was also associated with all-cause mortality in non-diabetic and diabetic subjects [39].

There is a strong genetic influence of A1C. Genes influencing A1C are in metabolic pathways influencing glycemic status, red blood cells and others – including FN3K which catalyzes the deglycation of glycated hemoglobin [23,32]. In non-diabetic subjects, 62% of population variation in A1C is explained by genetics and the remainder is attributable to the influence of environment (23%) and age (14%). Genetic influence of A1C also extended in patients with diabetes [40]. A1C may underestimate the glycemic status of subjects with sickle cell anemia and patients with renal failure on dialysis [30,31] (Table 4).

3. Glycated albumin and serum fructosamine

Glycation of serum albumin by glucose produces glycated albumin (GA) which is also a clinical biomarker of glycemic control. It is increasingly preferred where interferences in A1C apply – see above. The major sites of glycation are, in order of reactivity: asp-1, lys-525, lys-199 and lys-439 [41]. The extent of albumin glycation in healthy human subjects is 11–16%, increasing 2–3 fold in patients with diabetes [42]. Albumin has a half-life of *ca.* 20 days, and provides a report on glucose control over 14–20 days prior to blood sampling [43]. GA shows 48% genetic influence in population variation [44,45] (Tables 3 and 4).

Albumin is secreted into plasma by the liver. It leaks from the vascular into interstitial fluid at 5% of total plasma pool per h – the albumin transcapillary escape rate (TER). It returns to plasma via lymph. Albumin leaking through renal glomeruli is returned to the venous circulation by the renal albumin retrieval pathway where GA may be preferentially excreted [6,46,47] (Fig. 1c). Given the decreased concentrations of albumin and glucose in the interstitial fluid compared to plasma, the rate of glycation of albumin by glucose is *ca.* 4-fold lower in the interstitial fluid compared to plasma (Fig. 2a) [48]. Change in albumin TER in hypertension, obesity, diabetes, acute inflammatory disease and peripheral artery disease (PAD) may provide an interference in use of GA as a biomarker of glycemic control [49–54]. Decreased synthesis, plasma concentration and catabolism of albumin in patients with cirrhosis likely explains the 2-fold increase of GA without change in glycemic status [10,46]. It was initially proposed from experimental studies that AGE-modified albumin is extracted from plasma by the liver [55]. Clinical studies of levels of endogenous AGE-modified albumin in hepatic artery, portal vein and hepatic vein did not support this [10]. Albumin ligands used in experimental studies had high, supra-physiological extents of glycation and abnormally high increased net negative charge which bind hepatic scavenger receptors for cellular uptake [55–57]. Endogenous AGE-modified albumin clinically or albumin modified by AGEs *in vitro* to physiological extent showed no binding of the scavenger receptor and limited extraction in the liver [10,58,59]. The cellular uptake and proteolysis of FL- and AGE-modified albumin is rather proposed to occur in multiple tissues, similar to that of unglycated albumin [10]. Recent studies suggest glycated proteins are directed for cellular proteolysis by the unfolded protein response [60,61].

A further interference is through effects of renoprotective drug treatment on leakage of albumin through the renal glomerular filter (Fig. 1c). Patients with early-stage diabetic nephropathy, microalbuminuria, are treated with angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs). This decreases the size of the pores of the renal glomeruli filter, decreasing leakage of albumin and particularly GA into the glomerular filtrate. Treatment with ARB, Irbesartan, increased in GA by 66–72% without change in A1C [62].

A glycation biomarker related to GA is serum fructosamine – a measure of total serum protein glycation by glucose. It is determined as the rate of reduction of serum protein using nitroblue tetrazolium (NBT) at pH 10.8 [63], reports on glycemic control over 10–14 days prior to sampling [43] and likely has similar interferences to GA. The concentration of serum fructosamine in healthy human subjects was 230 ± 26 μM [64] but the absolute calibration is challenging; different fructosamines reducing NBT at different rates [63,65]. Measurement of serum fructosamines or "Fructosamine test" is low cost and in widespread clinical use.

Both serum fructosamine and GA correlate positively with A1C [64]. Cohen et al. introduced a derivative variable called the glycosylation gap (GG) which is the difference between measured A1C and A1C predicted from the fructosamine based on the A1C-fructosamine regression equation [66]. GG may predict progression of diabetic nephropathy [67]. From the above considerations, increased albumin TER and renoprotective drug treatment may produce negative and positive GGs, respectively [48,62]. GG also has strong genetic influence (Table 4).

4. Glycated skin collagen and other fructosamine-modified proteins

FL residue content of skin collagen increases with age of healthy

Table 2
Major analytical methodologies for glycation biomarkers in clinical practice and research. Steps to clinical translation.

MAJOR ANALYTICAL METHODOLOGIES			
Glycation analyte	Analytical methodology	Comment	Reference
Clinical practice application			
Glycated hemoglobin HbA_{1c}	Immunoassay (recognizing glycated N-terminus of β-chain).	Rapid and high sample throughput. Automated in clinical chemistry analyzers.	[129]
	Cation exchange chromatography with absorbance spectrophotometric detection	Moderate sample throughput. Automated in clinical chemistry analyzers. Interference from rare hemoglobin variants.	[130]
Glycated hemoglobin HbA_1	Boronate affinity chromatography with absorbance spectrophotometric detection	Moderate sample throughput. Measures sum of HbA_{1a}, HbA_{1b} and HbA_{1c}. Automated in clinical chemistry analyzers. Minimal interference from hemoglobin variants.	[131]
Glycated albumin	Enzymatic chromogenic assay (ketoamine oxidase/peroxidase)	Moderate sample throughput. Requires prior processing of glycated amino acids followed by proteolytic digestion.	[132]
Glycated albumin	Boronate affinity chromatography with absorbance spectrophotometric detection	Moderate throughput. Measures percentage albumin with fructosamine residues	[133]
Serum fructosamine	Absorbance spectrophotometry	Rapid and high sample throughput. Automated in clinical chemistry analyzers. Interferences: hemoglobin, bilirubin, glutathione, cysteine.	[63,134]
Research application			
All glycation analytes	Stable isotopic dilution analysis LC-MS/MS	Reference method. Moderate throughput. Multiplexing at minimal additional cost and without interference. Prior enzymatic hydrolysis for glycated proteins. Not yet fully automated. Limited commercial availability of analytical standards.	[9,14,96]
All glycation analytes	Immunoassay	High throughput. Different response for glycation adduct residues of proteins and glycation free adducts, requiring separation in pre-analytic processing. Often incomplete characterization of epitope specificity and calibration standards. Masked epitopes and other sample matrix effects. Interference from analyte in blocking proteins – advise use of polythreonine. Expensive to multiplex and risk of interference. Limited commercial availability of specific antibodies other than for CML. Preferably corroborated by LC-M/MS.	[92, 135–137]
Fluorescent AGEs	HPLC with fluorimetric detection	Medium throughput. Prior enzymatic hydrolysis for glycated proteins. Limited commercial availability of calibration standards. Can be run in-line with LC-MS/MS.	[9,14,96]
	Fluorimetry: excitation 350 nm, emission 440 nm "AGE fluorescence".	Multiple fluorophores. Interference from non-AGE fluorophores. Excitation 328 nm, emission 378 nm wavelengths have also been used.	[68,138]

STEPS TO CLINICAL TRANSLATION OF PROTEIN GLYCATION BIOMARKERS – including application of machine learning to develop classifier algorithms.	
Step	Requirements
1. Analytical method development and standard operating procedure.	Analytical sensitivity and specificity – including investigation of interferences; linearity and dynamic range; internal and calibration standards; limits of detection and quantification; intrabath and interbatch coefficients of variation; conditions for clinical sample collection, storage and pre-analytic processing; protocol for data analysis and quality control.
2. Application to clinical studies.	Determine reference interval in control study group.
3. Development of classifier algorithms.	Assay protein glycation biomarkers in case and control study groups, with power analysis for appropriate sample number.[a] Train and test algorithms with analytical data from independent subject groups, optimizing for accuracy of case and control classification.
4. Further validation studies.	Applicability to subjects of different age, gender, ethnicity and effect of co-morbidities and treatment. Algorithms for diagnosis, risk of severity progression and response to therapy of a health disorder or disease.

[a] An introduction to power analysis in clinical diagnostics was given by Xia et al. [139].

subjects, increasing *ca.* 45% from 20 to 80 years of age [68]. This may reflect age-dependent decrease of glucose tolerance [69]. There was no age-dependent link to skin collagen FL in patients with T1DM, although levels correlated positively with A1C and were decreased with therapeutic improvement of glycemic control [68,70]. In patients with T1DM of the Diabetes Control and Complications Trial (DCCT), increased skin collagen furosine was associated with worsening of diabetic retinopathy, nephropathy and neuropathy [71]. A similar study explored association with measures of subclinical CVD, finding furosine predicted future increased coronary artery calcification (CAC) and change of left ventricular mass/end diastolic volume ratio [72]. This may reflect the progression of CVD with increased hyperglycemia [37,38].

Other fructosamine-modified proteins studied are glycated low density lipoprotein (LDL) and high density lipoprotein (HDL). They are investigational risk predictors of atherosclerosis and coronary heart disease (Table 3).

5. Proteins modified by advanced glycation endproducts

Proteins modified by AGEs are currently investigational clinical biomarkers, typically measured in plasma protein and skin collagen. In a validation study for application to epidemiological investigations, plasma protein GSP and MG-H1 showed the highest reliability in replicate samples and greatest stability with delayed processing and storage [73].

In healthy subjects, plasma protein GSP correlated positively with subject age [73]. In skin collagen, CML, CEL, MG-H1, GSP, pentosidine and "AGE fluorescence" (excitation 328 nm, emission 378 nm) correlated positively with age in healthy subjects [68,74]. GSP is a stable degradation product of FL and may provide an improved cumulative measure of glucose exposure and dysglycemia, relative to FL [75]. It is the major glycation crosslink in skin collagen and may contribute to age-related impairment of elastic properties in skin [76]. MG-H1 has a half-life of only 12 days under physiological conditions so its increase with age is likely reflecting exposure to increased MG or dicarbonyl stress with age [77,78].

In patients with T1DM, plasma protein CML correlated positively with A1C and mean 24 h plasma glucose, consistent with glycemic control biomarker FL being often the major precursor of CML [4]. Increased plasma protein CML and CEL were positively associated with arterial pulse pressure [79], increased CML, CEL and pentosidine were associated with increased risk of CVD and all-cause mortality [80] but CML and other AGEs were not linked to the risk of developing diabetic nephropathy [81,82]. In skin collagen, glycation-related analytes including CML, CEL, G-H1, GSP, MG-H1 and pentosidine were risk predictors of progression of retinopathy and neuropathy but not nephropathy, with GSP and MG-H1 major contributors [71]; and CML, MG-H1, GSP and pentosidine also predictors of surrogate clinical endpoints of CVD [72].

In patients with T2DM, an increased AGE score computed from

Table 3
Glycated proteins and free adducts as clinical biomarkers.

Health decline or disease (Type of biomarker)	Glycation analyte (adduct)	Diagnostic indication and biomarker characteristics	Reference
Proteins with early-stage glycation by glucose			
Diabetes and prediabetes (Diagnostic biomarker and therapeutic monitoring - glycemic control)	Glycated hemoglobin A1C (fructosamine)	Diagnosis: normal, A1C <5.7% (<39 mmol/mol); prediabetes, A1C 5.7–6.4% (39–47 mmol/mol); diabetes, A1C ≥ 6.5% (≥48 mmol/mol). Therapeutic monitoring (glycemic control): good, <7% (53 mmol/mol); moderate, <8% (53 mmol/mol); poor, ≥8% (53 mmol/mol). In widespread clinical use.	[15,16,19]
	Glycated albumin (fructosamine)	Diagnosis: normal glycemia 11–16%. In widespread clinical use, particularly where there are interferences in use of A1C. Likely interference from change in albumin synthesis, catabolism, TER and glomerular filtration.	[42,140]
Microvascular and macrovascular complications of diabetes (Risk predictor)	Glycated skin collagen (fructosamine)	Risk prediction: increased furosine linked to worsening of diabetic retinopathy, nephropathy and neuropathy and increased CAC and left ventricular mass/end diastolic volume ratio.	[71,72]
Cardiovascular disease (Risk predictor)	LDL (fructosamine)	Apolipoprotein B100 major protein; half-life 3 days. Glycated in 6 domains. Difficult to quantify at all sites. May be risk predictor of myocardial infarction and surrogate of small dense LDL	[141–145]
	HDL (fructosamine)	Apolipoprotein A-1 major protein; half-life 3.3 days. 3–5% glycation; sites - lys-12, lys-96, lys-133, lys-205 and lys-239 Difficult to quantify. May be surrogate marker of HDL instability	[91, 146–148]
Advanced glycation endproducts-modified proteins			
Healthy aging (Diagnostic marker)	Plasma protein (GSP)	Correlates positively with age.	[73]
	Skin collagen (CML, CEL, GSP, MG-H1, pentosidine)	AGEs increase with donor age. May be linked to skin aging and age-linked decline in glucose tolerance and dysglycemia	[68,74]
Diabetes (Diagnostic marker; glycemic control)	Plasma protein (CML, GSP)	CML correlated positively with A1C and mean 24 h plasma glucose. Plasma protein GSP increased with T2DM.	[4,73]
Microvascular and macrovascular complications of diabetes (Risk predictor)	Plasma protein (CML, CEL and pentosidine)	T1DM: Increased plasma protein AGEs associated with increased risk of CVD and all-cause mortality. T2DM: inverse link to PAD.	[80,83]
	Skin collagen (CML, CEL, G-H1, GSP MG-H1 and pentosidine)	Predicted risk of diabetic retinopathy, diabetic neuropathy and increased risk of CVD (CIMT, increased left ventricular mass, and increased LV mass/end diastolic volume ratio).	[71,72[71, 72]
CKD (Risk predictor)	Plasma protein and free adduct (pentosidine)	Risk predictor of all-cause mortality.	[87]
CVD (Risk predictor)	Plasma protein (MG-H1)	Increased mortality in nondiabetic women	[90]
Glycation endproduct free adducts			
Chronic kidney disease (Risk predictor)	Fractional excretion of free adducts (FL, CML, CEL MG-H1, CMA and Pentosidine)	Fractional excretion of glycation free adduct is increased in patients with diabetes at high risk of EDRF, supporting a precision medicine approach.	[8]
	Plasma free adducts (CML, CEL and MG-H1)	Logistic regression model for progression of diabetic kidney disease	[106]

Table 4
Influence of genetics on protein glycation.

Glycated protein(s)	Type of study	Observation	Reference
Glycated hemoglobin A1C	Twin study (non-diabetic subjects)	Population variation: 62% genetics; 23% environment; 14% age. Genetic influence extended to patients with T1DM.	[40]
	GWAS study (non-diabetic subjects)	60 loci influencing A1C, explaining 4%–14% of the trait variance: 19 genes of glycemic status, 22 genes of red blood cell pathways, 19 genes unclassified.	[32]
Glycated albumin GA	First degree relatives and GWAS (non-diabetic subjects)	Population variation: 48% genetics. Associated SNP: GCKR - SNPs identified accounted for 3.2% of the variation.	[44,45]
Glycation gap (GG)	Twin study (non-diabetic subjects)	Population variation: 69% genetics; 31% environment. Twenty percent heritability of A1C was common to GG.	[149]
Serum protein CML	Twin study (non-diabetic subjects)	Population variation: 74% genetics.	[150]

plasma protein CML, CEL and pentosidine was inversely linked to BMI, risk of PAD and GFR [62,83]. These associations may be due to increased albumin TER in obesity and PAD and increased loss of AGE-modified albumin through the glomerular filter with increased GFR [50,54]. Increased MG-H1 residue content of serum protein was associated with diabetic retinopathy in patients with T2DM [84]. Plasma protein CML was inversely associated with BMI in a large cohort of obese and insulin resistance subjects – some with T2DM [85], which may be due to increased albumin TER in obesity and insulin resistance [50–52] (Table 3).

Protein glycation biomarkers in CKD were recently reviewed elsewhere [86]. Total plasma pentosidine (protein-bound + free adduct), was a risk predictor of mortality in CKD after adjusting for all confounders [87] and may be reporting increased pentosephosphate pathway activity in response to oxidative stress induced by rapid decline in renal function and associated increased mortality [8]. CML residue contents of plasma protein was investigated as a biomarker of mortality risk in renal failure in patients receiving hemodialysis therapy in two studies with contrary outcomes [88,89]. Whilst formation of AGEs likely has a role in the etiology of renal disease [86], there is interference in application of plasma protein glycation biomarkers from changes in albumin synthesis and protein loss in dialysis.

For CVD, plasma protein MG-H1 was associated with increased mortality in nondiabetic women [90]. These observations suggest dicarbonyl stress may have a key role in CVD, particularly through formation of MG-modified small, dense LDL and MG modified HDL with decreased stability and half-life in pro-atherogenic dyslipidemia [91, 92]. Supporting this, a genome wide association study found expression of glyoxalase 1 (Glo1) which metabolizes MG was negatively linked to risk of coronary heart disease [93]. Increased plasma protein CML and pentosidine were associated with poor diastolic and systolic heart function [94], which may be due to interference of decreased albumin

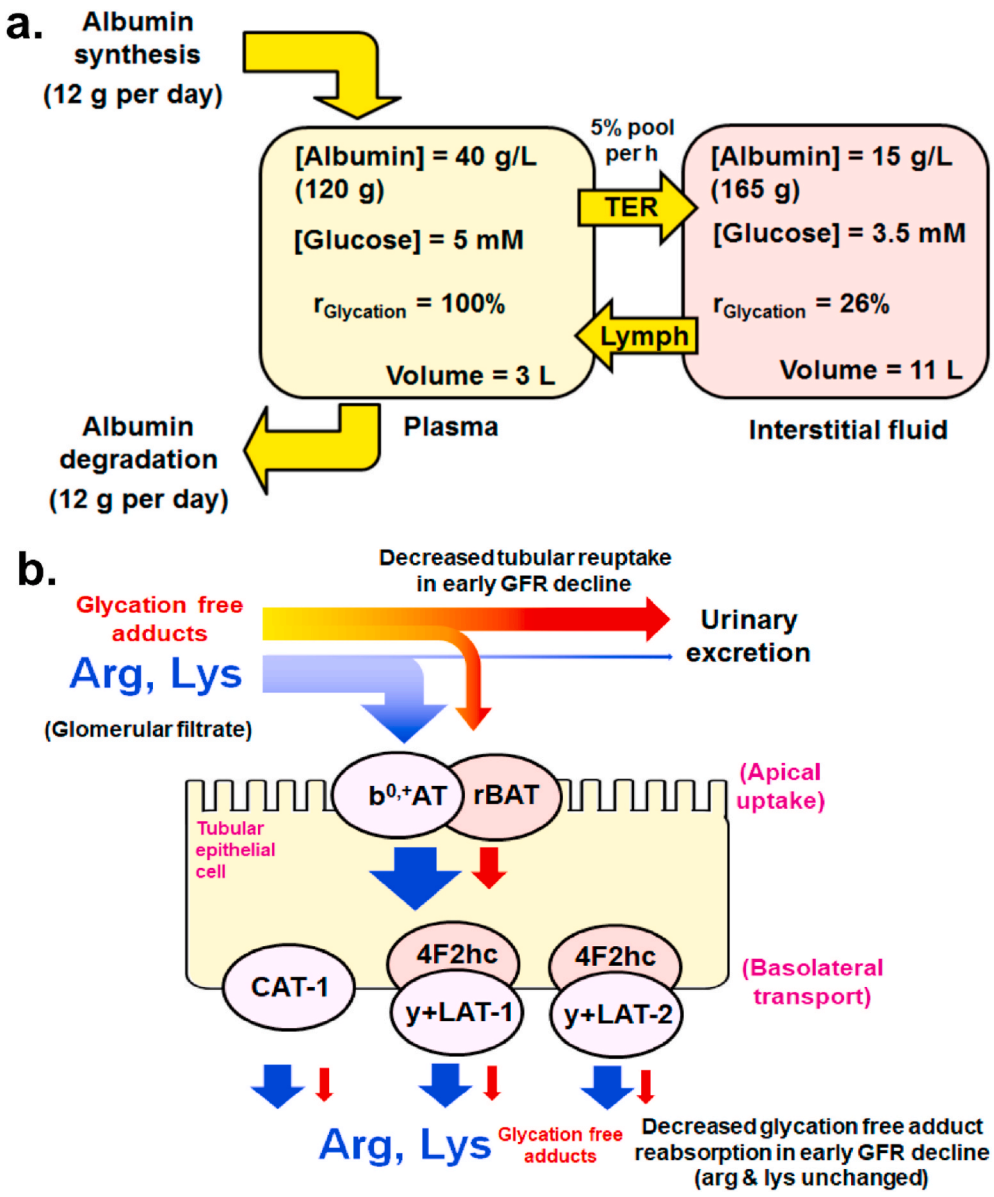

Fig. 2. ***Compartmentalization of albumin glycation in plasma and interstitial fluid and impaired renal tubular reuptake of glycation free adducts in early-stage diabetic kidney disease.*** **a.** Glycation of albumin by glucose – glycation kinetics and dynamics in vascular and extravascular compartments. Physiological data from Refs. [6,152]. Relative glycation kinetics deduced from: $r_{Glycation} = k$ [Glucose][albumin], k is the glycation rate constant and assuming $r_{Glycation}$ in the plasma compartment = 100%. Albumin TER is increased (5.6–7.6% per h) and plasma volume decreases by up to 10% in hypertension and overweight/obese subjects with metabolic syndrome; obesity increases total body interstitial fluid volume [49,50,153]. **b.** Schematic diagram of amino acid transporters of arginine and lysine uptake in the renal tubular epithelium and engagement with glycation free adducts. Renal proximal tubular reuptake by low affinity binding to cation transporter proteins: heterodimeric complex $b^{0,+}$AT/rBAT on the apical surface (gene names SLC7A9 and SLC3A1, respectively) and high affinity cationic amino acid transporter 1 (CAT-1) and heterodimeric complexes of y + LAT1, y + LAT2 with 4F2hc on the basolateral surface (gene names SLC7A1, SLC7A7, SLC7A6 and SLC3A2, respectively) [99–103]. Reproduced from Refs. [8,48] with permission. Abbreviations: GRF, glomerular filtration rate; TER, transcapillary escape rate.

TER [95].

6. Glycation free adducts

Assay of glycation free adducts requires minimal pre-analytic processing and is robust with short analysis time using stable isotopic dilution analysis LC-MS/MS [9,14]. Increased glycation free adducts in plasma and urine may be biomarkers of increased exposure to protein glycation and/or increased proteolysis of long-lived extracellular matrix proteins that have accumulated long-lived, chemically stable glycation adducts throughout life [96]. Recent studies suggest renal handling of glycation free adducts provides a very sensitive measure of patients who develop rapid loss of renal function or "early decline in renal function" (EDRF).

Diabetic kidney disease (DKD) occurs in about 40% patients with diabetes [97]. In a subset of patients with EDRF, renal function declines rapidly such that after 5–20 years, patients require renal dialysis and with median survival thereafter of only 3 years. During renal function decline, there is also a progressive increase in risk of fatal CVD, 3 to 20-fold higher than the healthy population. It is estimated that 19% patients with T1DM and 28% patients with T2DM develop EDRF [98].

Improved treatment and care could be provided if patients at risk of future development of EDRF could be identified. In an investigation of glycation free adducts in plasma and urine of patients with T1DM, with and without future development of EDRF (assessed during 12 years of follow-up), we found urinary excretion of pentosidine was increased in patients with EDRF, compared to patients with stable renal function. Remarkably, fractional excretions of 6 lysine and arginine-derived glycation free adducts were higher in patients with EDRF, compared to patients with stable renal function. Lysine and arginine-derived glycation free adducts are thought to be taken up and moved across the renal tubular epithelium by low affinity binding to cation transporter proteins which also take up arginine and lysine. These are: heterodimeric complex $b^{0,+}$AT/rBAT on the apical surface and CAT-1 and heterodimeric complexes of y + LAT1, y + LAT2 with 4F2hc on the basolateral surface [99–103]. Interestingly, in genome-wide association studies genetic polymorphism of y + LAT1, y + LAT2, $b^{0,+}$AT (genes SLC7A7, SLC7A6, and SLC7A9) was associated with variation in eGFR and development of CKD [104,105]. Impaired tubular reuptake of glycation free adducts by lysine and arginine transporter proteins in patients with EDRF is likely involved (Fig. 2b). Increased fractional excretions of glycation adducts are potential risk predictors for EDRF in diabetes and possibly

progression of non-diabetic CKD [8]. In an alternative approach, a logistic regression model indicated that plasma concentrations of CML, CEL and MG-H1 free adducts, but not A1C, were linked to progression of diabetic kidney disease in patients with T1DM, assessed by thickening of the renal glomerular basement membrane [106] (Table 3).

7. Application of artificial intelligence machine learning for the development of diagnostic algorithms with protein glycation features

Combination of multiple measurements as features in trained and tested algorithms is a recently developed strategy to improve clinical diagnostic performance of glycation adduct biomarkers. Plasma protein content of AGEs, CML, CMA, 3-deoxyglucosone-derived hydroimidazolone (3DG-H) and oxidative damage marker, dityrosine (DT), provided features in a diagnostic algorithm for autism spectrum disorder (ASD) or autism [101]. Autism is a developmental disorder of childhood that affects 12 million people worldwide with a global prevalence of 0.62% [107], with higher prevalence in the USA (2.47%) [108] and Europe (1.15%) [109]. Plasma protein contents of CML, CMA and DT were higher and plasma protein content of 3DG-H lower in children with ASD, compared to children with normal development. A diagnostic algorithm combining these analytes gave a test with 88% accuracy. An important indicator of diagnostic performance is positive likelihood ratio LR+, which is the ratio of subjects with a positive test who have a health disorder or disease, compared to number of subjects with a positive test who do not. Similarly, negative likelihood ratio LR− is the ratio of subjects with a negative test who have a health disorder or disease, compared to number of subjects with a negative test who do not. LR+ >10 and LR− <0.1 is considered strong, often conclusive evidence for positive and negative diagnosis, respectively; and LR+ of 5–10 and LR− of 0.1–0.2 provides moderate evidence of positive and negative diagnosis, respectively [110]. For the autism blood test, LR+ = 5.7 and LR− = 0.095, suggesting moderate and strong, often conclusive evidence of presence and absence of ASD, respectively [101]. Regarding algorithm features, increased CML and CMA residues in plasma protein may reflect increased plasma protein glycation by glyoxal, sourced mainly from lipid peroxidation [14]. Recent studies have associated genetic polymorphism of fatty acid desaturase (FADS) 1/2 and elongation of very long-chain fatty acids protein-2 (ELOVL2) with risk of ASD [111]. This may indicate a disturbance of long chain polyunsaturated fatty acid metabolism with increased lipid peroxidation in ASD. DT residue content of plasma proteins, a marker of protein oxidative damage, was increased in subjects with ASD whereas other oxidative damage markers were not. DT residue formation occurs by reaction of tyrosine residues in proteins with ROS and dual oxidase DUOX [112]. DUOX has an important role in gut mucosal immunity, host–microbe homeostasis and signaling for neutrophil recruitment into allergic airways [113]. Gut microbiota may be influential in development of the behavioral phenotype in ASD [114]. Decrease of 3DG-H content of plasma protein in subjects with ASD may reflect decreased concentration of plasma 3-DG due to increased 3-DG reductase activity, as discussed [101]. With further validation, this may provide the basis of a simple blood test for autism diagnosis. This is an example of combination of biomarkers of protein glycation and oxidative damage.

Application of glycation free adducts for clinical diagnosis is best developed for diagnosis and classification of early-stage arthritis. Protein glycation and oxidative damage accumulates in articular cartilage with age [115–117]. From the earliest stages, pathogenesis of arthritis involves increased proteolysis of articular cartilage. There are three main types of arthritis clinically: osteoarthritis (OA), rheumatoid arthritis (RA) and other inflammatory joint disease which is often self-resolving (non-RA). Arthritis type is often difficult to discern at the early stages. Early-stage detection may enable effective treatment of RA with disease-modifying anti-arthritic drugs and OA with beneficial lifestyle and therapeutic interventions. Early-stage arthritis was detected and type assigned with diagnostic algorithms based on features of plasma glycation, oxidation and nitration free adducts in combination with hydroxyproline. LR+ and LR− for early-stage diagnosis was 10.2 and 0.09 respectively, indicating conclusive evidence for presence or absence of early-stage arthritis (any type). The type of arthritis could also be discerned where anti-citrullinated cyclic peptide (CCP) antibody positivity status was a feature [118]. This illustrates how combination of protein glycation, oxidative and nitration damage markers with the bone resorption biomarker, hyp, and anti-CCP antibody status – a clinical RA-biomarker requiring refinement, may provide high performance diagnosis.

A urinary health screen with age, BMI, FL free adduct and valine as features was also recently developed providing moderate, weak and strong evidence for early-stage decline in metabolic, vascular and renal health respectively (Table 5).

Table 5
Application of artificial intelligence machine learning for the development of diagnostic algorithms involved glycation adducts.

Disorder or disease (Type of biomarker)	Analytes (adduct)	Diagnostic indication	Reference
Autism spectrum disorder (Diagnostic marker)	Glycated plasma protein (CML, CMA, 3DG-H and DT)	Combined in a diagnostic algorithm, gave moderate evidence for presence and borderline moderate/conclusive evidence for absence of ASD; LR+ = 5.7, LR− = 0.095.	[101]
Early-stage arthritis (Diagnosis marker)	Plasma free adducts (FL, CML, CEL, G-H1, MG-H1, 3DG-H, CEL, CMA, GSP, pentosidine; and MetSO, DT, NFK, 3-NT; and hyp and anti-CCP antibody status)	Diagnostic algorithm for early-stage arthritis (any type) vs good skeletal health: LR+ = 8.3 and LR− = 0.11. Diagnostic algorithm for classification of early-stage arthritis type (OA, RA or non-RA): for OA, RA and non-RA, LR+ = 16.1, 7.7 and 5.0 and LR− = 0.06, 0.34 and 0.36, respectively.	[116]
Early-stage decline in metabolic, vascular and renal health.	Urinary free adduct (FL; and val, age and BMI)	Diagnostic algorithm classifying good health vs early-stage health decline. LR+, 8.0.2.8 and 13.2, and LR− 0.24, 0.43 and 0.13 for metabolic, vascular and renal health respectively.	[151]

Interpretation of level of evidence from likelihood ratios: LR+: 1–2, minimal; 2–5, small; 5–10, moderate; >10, large and conclusive. LR−: 0.5–1.0, minimal; 0.2–0.5, small; 0.1–0.2, moderate; <0.1, large and conclusive [110].

8. Overview and concluding remarks

A1C, GA and serum fructosamine are important clinical biomarkers in widespread use for assessment of glycemic control in patients with diabetes and A1C for also diagnosis of prediabetes and diabetes. Measurement of AGE residue content of skin collagen may provide an assessment of aging of skin and healthy aging, an indicator of glycemic control and risk prediction of vascular complication of diabetes. These are valuable clinical biomarkers because they reflect pathways mechanistically linked to the health disorder and disease phenotype: fructosamine, GSP and CML - hyperglycemia and glycemic control; MG-H1, CEL and G-H1 - dicarbonyl stress and metabolic dysfunction of unscheduled glycolysis linked mechanistically to the development of

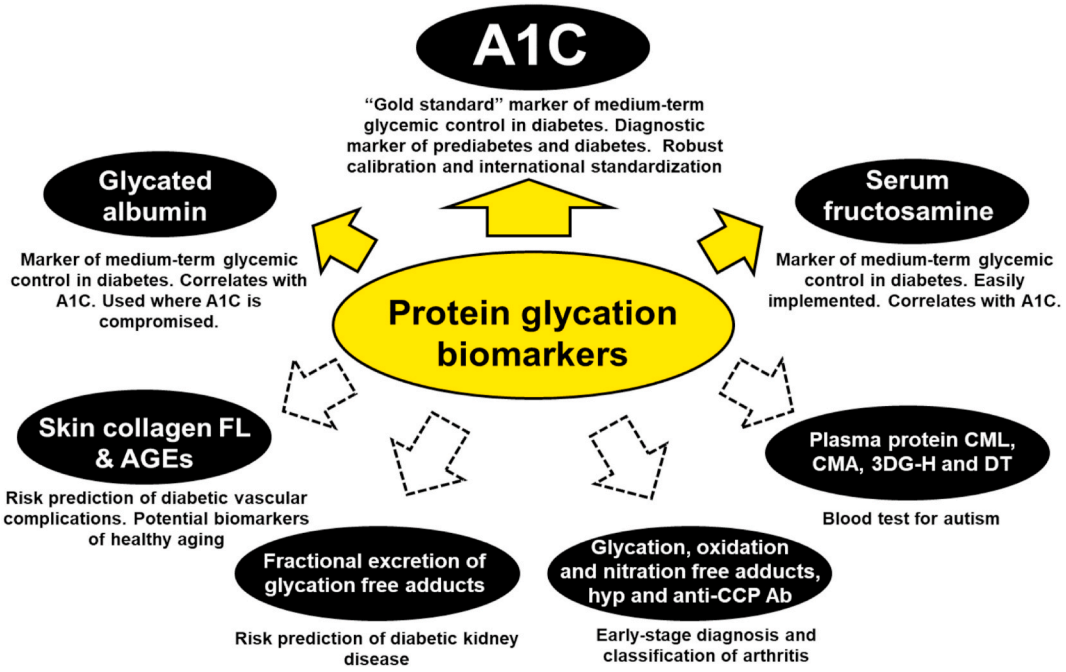

Fig. 3. ***Biomarkers of glycation in clinical use and investigational development.*** See text for details. Key: solid line bordered filled arrows – glycation biomarkers in clinical use; dotted line bordered unfilled arrows - glycation biomarkers in investigational development. Abbreviations: A1C, glycated hemoglobin HbA_{1c}; anti-CCP Ab, anti-cyclic citrullinated peptide antibody; CMA, N□-carboxymethyl-arginine; 3DG-H, 3-deoxyglucosone-derived hydroimidazolone; and DT, dityrosine.

T2DM and diabetic vascular complications; and pentosidine - increased pentosephosphate pathway activity [9,28,119] (Table 1). Increased plasma glycation, oxidation and nitration free adducts combined with hyp and increased fractional excretion of glycation free adducts in diabetes with microalbuminuria may also be valuable diagnostic biomarkers of early-stage arthritis and risk predictors of DKD because they are close to the early-stage disease phenotype – increased joint proteolysis and decline in renal tubular function, respectively. Such biomarkers are likely to provide valuable diagnostic information and support a precision medicine approach. Protein glycation biomarkers are influenced by heritability, aging and decline in metabolic, vascular, renal and skeletal health and other factors. They are therefore applicable to populations of differing ethnicities, bridging the gap between genotype and phenotype [120], and thereby likely to find continued and expanding clinical use. A summary of established and emerging applications of protein glycation biomarkers is given: established biomarkers of glycemic control – A1C, glycated albumin and serum fructosamine; emerging biomarkers of risk prediction of vascular complications of diabetes, early-stage detection and classification of arthritis, a blood test for diagnosis of autism and, potentially, biomarkers of healthy aging (Fig. 3). For future research, with increased availability of analytical standards and LC-MS/MS assay platform for use or reference validation of immunoassays, and application of machine learning algorithm development for optimum biomarker combination and weighting, we call on collaborative efforts from the glycation research community with custodians of large clinical sample collections and computational scientists to develop and validate emerging and further innovate applications of protein glycation biomarkers for clinical diagnosis and screening, disease progression and therapeutic monitoring.

Declaration of competing interest

The authors declare that they have no conflict of interest in relation to the above manuscript.

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