

Review

Importance of measuring products of non-enzymatic glycation of proteins

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Abstract

Non-enzymatic glycation products are a complex and heterogeneous group of compounds which accumulate in plasma and tissues in diabetes and renal failure. There is emerging evidence that these compounds may play a role in the pathogenesis of chronic complications associated with diabetes and renal failure. So measurement of the products of non-enzymatic glycation has a twofold meaning: on one hand, measurement of early glycation products can estimate the extent of exposure to glucose and the subject's previous metabolic control; on the other hand, measurement of intermediate and late products of the glycation reaction is a precious instrument in verifying the relationship between glycation products and tissue modifications. This review summarizes current knowledge about the diagnostic utility of measuring non-enzymatic glycation products.

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Keywords: Non-enzymatic glycation products; Amadori product; Advanced glycation end-products (AGE); Diabetes; Renal failure; Chronic complications

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Abbreviations: ADA, American Diabetes Association; AGE, Advanced Glycation End-Products; CAPD, continuous ambulatory peritoneal dialysis; DCCT, Diabetes Control and Complications Trial; CML, carboxymethyl lysine; EDIC, Epidemiology of Diabetes Interventions and Complications; EGF, Endothelial Growth Factor; ELISA, Enzyme Linked Immunosorbent Assay; GC/MS, Gas Chromatography/Mass Spectrometry; HD, hemodialysis; HPLC, High Performance Liquid Chromatography; IFCC, International Federation of Clinical Chemistry; IGF1, Insulin-like Growth Factor 1; IgG, Immunoglobulin type G; LC/ESI/MS, Liquid Chromatography/Electrospray Ionization Mass Spectrometry; LDL, Low Density Lipoprotein; MALDI/MS, Matrix-Assisted Laser Desorption Mass Spectrometry; NBT, Nitro Blue Tetrazolium; NF- κ B, Nuclear Factor κ B; PDGF, Platelet Derived Growth Factor; RAGE, receptor for AGE; RCO, reactive dicarbonyl compounds; RIA, Radio Immunoassay; TNF- α , Tumor Necrosis Factor α ; VCAM, Vascular Cell Adhesion Molecule.

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Introduction

The non-enzymatic glycation of proteins, or Maillard reaction, is a process which links chronic hyperglycemia to a series of physiopathological alterations considered important in the development of the chronic complications of diabetes [1]. The Maillard reaction is subdivided into three main stages: early, intermediate, and late (Fig. 1). In the early stage, glucose (or other reducing sugars as fructose, pentoses, galactose, mannose, ascorbate, xylulose) react with a free amino group of several molecules, including proteins, nucleic acids, and lipids, to form an unstable aldimine compound, the Schiff base. Through rearrangement, this base gives rise to a stable ketoamine, the Amadori product. Since this reaction does not require the participation of enzymes, the variables which regulate it in vivo are

the concentrations of glucose and protein, the half-life of the protein, its reactivity in terms of free amino groups, and cellular permeability to glucose. In in vivo conditions, the Amadori product reaches equilibrium after approximately 15–20 days and, through irreversible links, accumulates on both short-lived and long-lived proteins.

In the intermediate stage, through oxidation and dehydration reactions, the Amadori product degrades into a variety of carbonyl compounds (glyoxal, methylglyoxal, deoxyglucosones) which, being much more reactive than the sugars from which they are derived, act as propagators of the reaction, again reacting with the free amino groups of proteins. In particular, methyl glyoxal is a very reactive alpha-oxaldehyde which may be formed both from reactions which depend on the levels of glucose (non-enzymatic glycation, the polyol pathway) and from intermediate

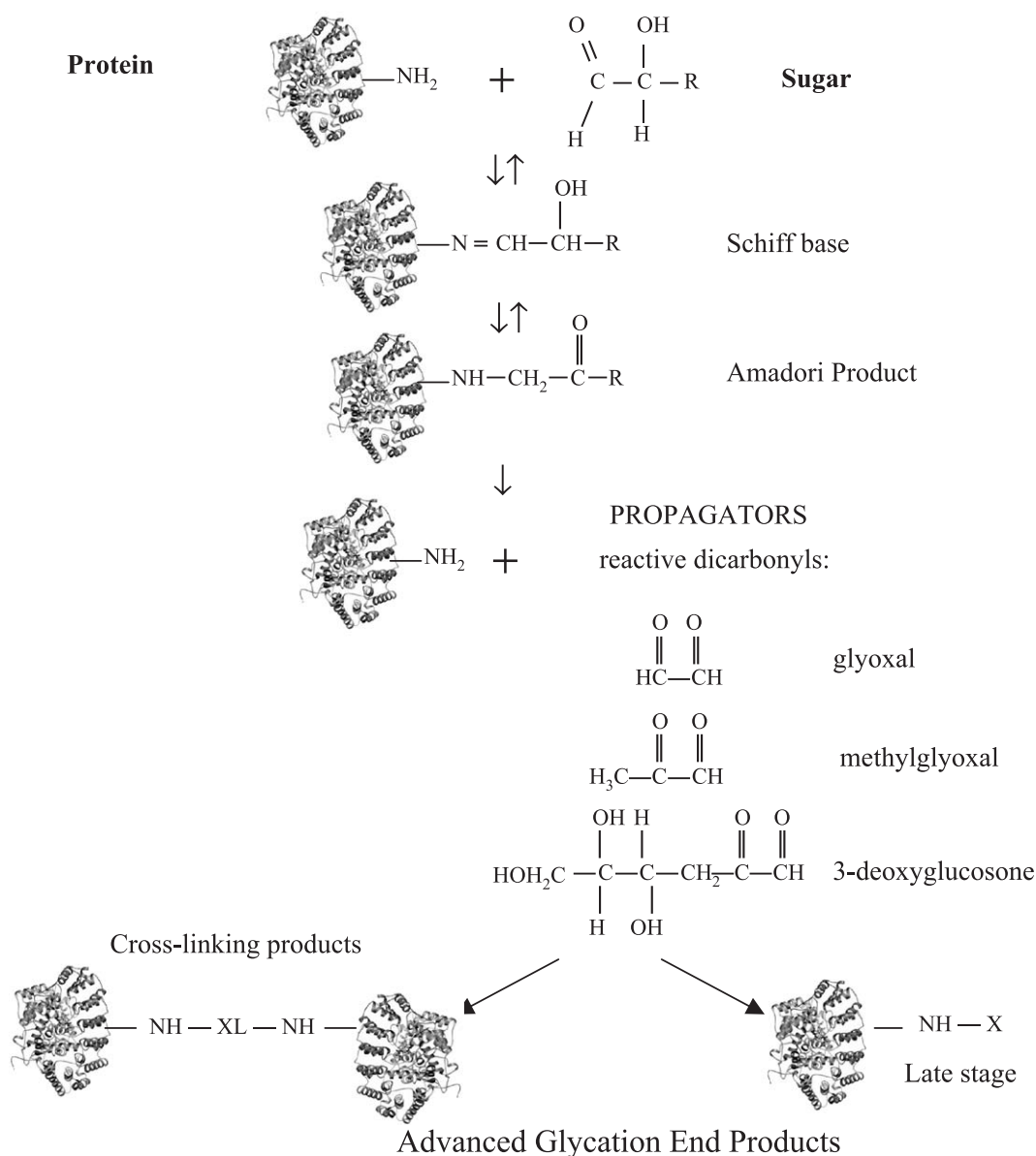


Fig. 1. The three steps of non-enzymatic glycation reaction.

products of glycolysis, metabolism of ketone bodies, and catabolism of threonine. The high reactivity and elevated plasma concentrations of methyl glyoxal indicate that this compound is one of the most important ones in vivo [2].

In the late stage, these propagators again react with free amino groups and, through oxidation, dehydration and cyclization reactions, form yellow-brown, often fluorescent, insoluble, irreversible compounds, usually called Advanced Glycation End-Products (AGEs), which accumulate on long-lived proteins and cause damage. Although the chemical nature of these compounds is not yet well-defined, recent investigations indicate that they include post-Amadori products derived from oxidation and further structural rearrangements, so that compounds which are neither cross-linked nor fluorescent have been considered to belong to the AGE group. In this context, it should be emphasized that oxidation processes are important in the formation of many AGEs [3]. There are two mechanisms through which these processes take place, both catalyzed by metals such as copper and iron. The first involves auto-oxidation of free sugar in the presence of oxygen and free metals, leading to more reactive dicarbonyl compounds, which react with proteins to form highly reactive ketoamines. The second mechanism involves protein-bound products of the Amadori pattern which, in the presence of oxygen and free metals, are oxidized and give origin to highly reactive protein-enediols and protein-dicarbonyls which can generate AGEs (Fig. 2).

The three main mechanisms by means of which AGEs cause tissue damage are: cross-link formation, interaction with specific cellular receptors, and intracellular glycation [4] (Fig. 3). In this context, one of the most important is the formation of abnormal and stable cross-links on collagen, as demonstrated both in *in vitro* incubation with glucose and *in vivo* in the collagen of diabetic patients. Glyco-oxidation causes several chemical and physical modifications of collagen, leading to high levels of absorbance (280 nm), fluorescence, increased resistance to enzymatic digestion

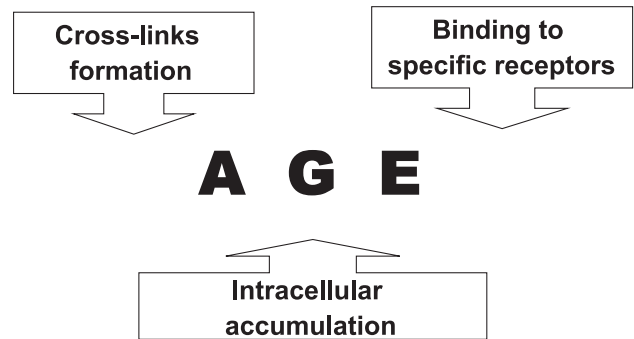


Fig. 3. AGE mechanism of damage.

and denaturants, increased thermal stability, and reduced solubility, all of which clearly explain some of the structural tissue modifications typical of the chronic complications of diabetes, such as abnormal vascular rigidity, arterial stiffness, and basement membrane thickness. Moreover, non-enzymatic glycation of collagen can inhibit the release of nitric oxide of endothelial derivation, with consequent vasoconstriction, reduced plasma flow, and tissue ischemia. Lastly, AGE compounds in collagen may trap multiple macromolecules such as lipoproteins, immunoglobulin, fibrin, and albumin. Immunoglobulins bound to collagen retain their ability to form antigen–antibody complexes which may be deposited on vessels. Increased glycated LDLs also bind covalently to glycated collagen, thus contributing to vessel occlusion. In this context, there are also other mechanisms which are involved in the formation of atherosclerotic plaques, which may cause vessel occlusion. First, glycated LDLs are not identified by their receptor, but are preferentially recognized by high-capacity, low-affinity scavenger receptors on monocyte/macrophages, enhancing uptake by them, with consequent stimulation of cholesterol synthesis and “foam cell” formation. Second, glycated LDLs are capable of stimulating thromboxane β_2 release and inducing platelet aggregation. Third, glycated

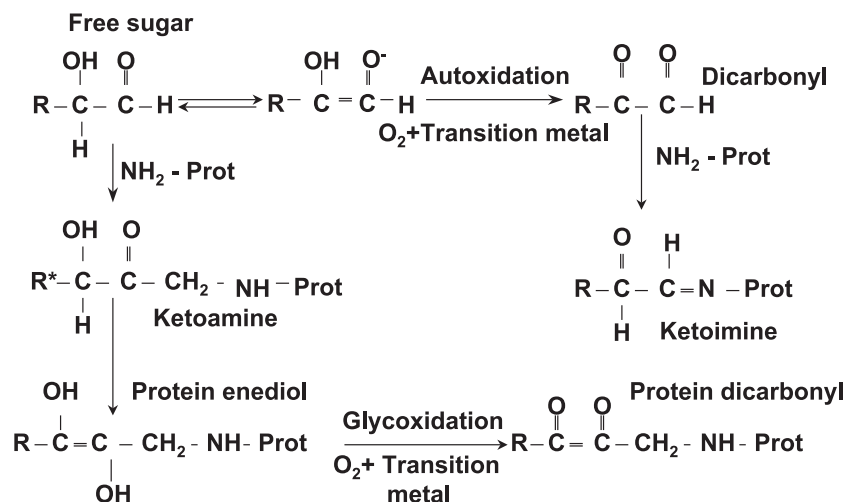


Fig. 2. Reaction schemes for glucose autooxidation and glycooxidation.

lipoproteins may generate free radicals, with consequent increased vessel oxidative damage. Lastly, glycated LDLs, due to their altered structure, are immunogenic and thus able to stimulate the production of antibodies: the resulting immune complexes may be deposited on vessel walls and stimulate “foam cell” formation.

Recent studies have shown the existence of specific cellular receptors which bind AGE proteins in a saturable manner. The first receptor for AGE, the “scavenger”, was purified from cell membranes and reported to have different molecular weights of 60 and 90 kDa. This receptor, expressed on the cell membrane of T lymphocytes and monocytes/macrophages, has the function of degrading senescent molecules. More recently, a 35-kDa protein, “RAGE”, has been identified as a new member of the Ig superfamily of cell surface molecules, codified from a gene on chromosome 6. Subsequently, this receptor was identified in various cell systems: monocytes/macrophages, T lymphocytes, fibroblasts, smooth muscle cells, neurons, red cells, and mesangial cells. The binding of AGE on RAGE T lymphocytes stimulates production of γ -interferon, with consequent tissue damage; binding of AGE to monocytes/macrophages induces production of cytokines (interleukin 1B, TNF- α , IGF-1, PDGF) and growth factors, with consequent increased synthesis of type IV collagen, increased proliferation of vessel smooth muscle cells, and stimulation of macrophage chemotaxis. In physiological conditions, cytokines are important regulators of tissue remodeling: on one hand, they stimulate the mesenchymal cells to produce hydrolase in order to degrade tissue proteins; on the other hand, they stimulate proliferation of endothelial and mesenchymal cells through reduction of tissue-derived growth factor. In diabetes, binding of AGE to RAGE reduces tissue protein degradation and increases production of growth factors,

with a consequent increase in the synthesis of the extracellular matrix and impairment of the mechanisms of tissue remodeling.

The damage induced by AGE–RAGE binding varies and depends essentially on the type of cells involved, since the AGE–RAGE interaction in fibroblasts and smooth muscle cells determines an increase in growth factors (EGF, PDGF), with consequent cell proliferation. AGE–RAGE binding on mesangial cells causes a series of alterations such as increased production of collagen IV, laminin, and fibronectin, and activation of PDGF, all of which explain some structural modifications characteristic of diabetic nephropathy. Lastly, through a mechanism of oxidative stress, AGE–RAGE binding on endothelial cells induces the transcription factor NF- κ B, which in turn increases expression of the vascular cellular adhesion molecule (VCAM-1). VCAM-1 overexpression then increases the adhesivity of monocytes to endothelial cells and vascular permeability, leading to accelerated transendothelial passage of AGE-modified proteins. Other alterations observed as a consequence of this AGE–RAGE interaction are an increase in the procoagulant response to TNF- α , a reduction in thrombomodulin expression, and an increase in endothelin-1 levels (Fig. 4).

Another protein with high binding affinity for AGE is galectin 3, later known as Mac-2 or 35 protein-binding carbohydrates, which is expressed on macrophages. The binding of AGE occurs at the COOH terminal peptide and promotes the formation of complexes of high molecular weight.

The third mechanism of AGE damage is dependent upon intracellular accumulation and has been demonstrated in macrophages, endothelial and smooth muscle cells, and atherosclerotic plaques. Two possible pathways of action are rapid intracellular formation of AGE induced by hyperglycemia, with consequent alteration of cytoplasmic and

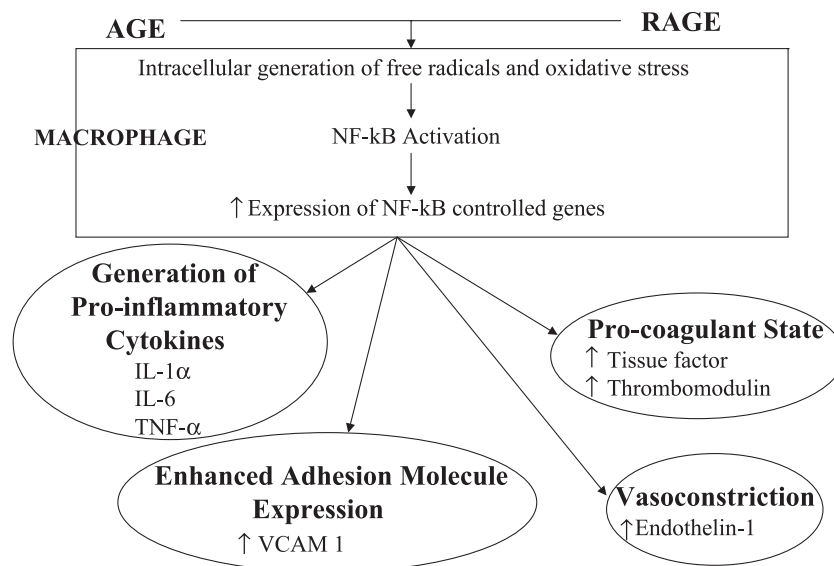


Fig. 4. AGE–RAGE interaction and NF- κ B activation leading to oxidant stress, vasoconstriction and a procoagulant state.

nuclear structures, and endocytosis, which follows binding to specific receptors.

An example of these three mechanisms of damage involved in the development of the chronic complications in diabetes is shown in Table 1, which highlights the role of AGE in diabetic atherosclerosis.

Measurement of products arising from the non-enzymatic glycation reaction therefore has a twofold meaning: on one hand, measurement of early glycation product, depending on the half-life of the glycated protein which is measured, yields an estimate of the extent of exposure to glucose and, therefore, the subject's previous metabolic control; on the other hand, measurement of the intermediate and late products of this reaction is a precious instrument in verifying the relationship between glycation products and tissue modifications, to clarify the pathogenesis of chronic complications and the association between exposure to glucose and its development (Table 2).

Measurement of early glycation product

Measurement of early-stage glycation product (Amadori product) is routinely used to evaluate metabolic control in diabetic patients. The two parameters commonly used are HbA1c and glycated serum proteins.

HbA1C

In adults, 97% of hemoglobin (Hb) is formed from HbA. In 1958, using ion exchange chromatography, Allen et al. [5] revealed three minor components of HbA, called HbA1a, HbA1b, and HbA1c, following the elution sequence. In 1968, Rahbar [6] noted that the smaller fractions, mainly HbA1c, were elevated in diabetic patients.

Subsequent studies have shown that HbA1c is derived from the non-enzymatic reaction between glucose and the

Table 2

Measurement of AGE: clinical applications

Physiopathological markers

Distribution, turnover, metabolism of AGE

Prognostic markers

- Serum AGE = chronic complications
- AGE HbA1c = long term glycemic control
- AGE LDL = atherosclerosis

Therapeutic markers

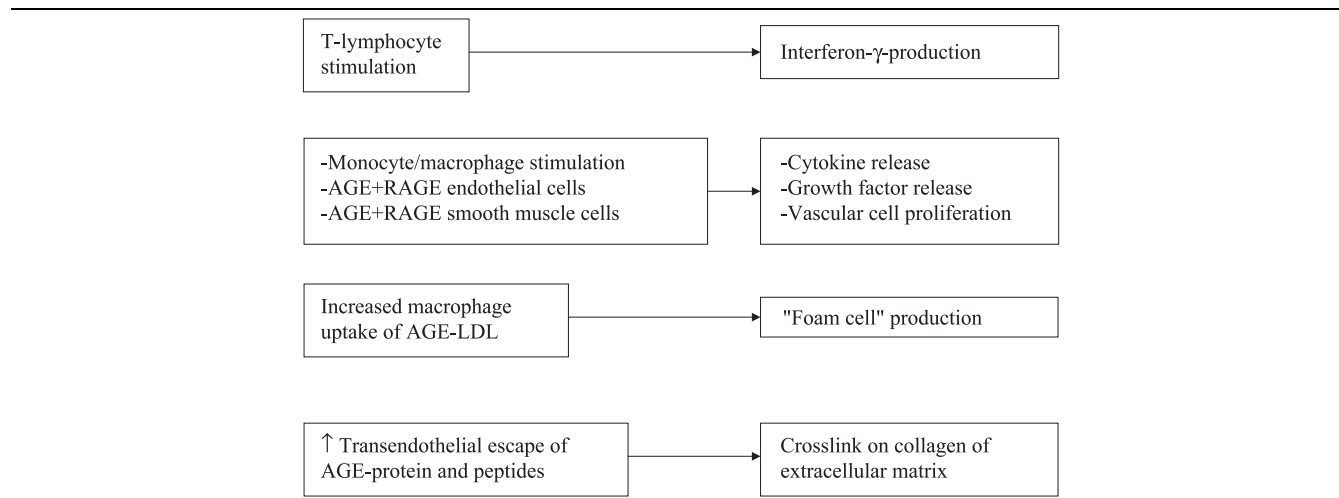
- Evaluation of the effect of AGE inhibitor
- Evaluation of the effect of renal replacement therapy

amino groups of valine and lysine of β -globin [7]. In 1978, the term *glycosylated hemoglobin* was introduced to indicate this non-enzymatic reaction [7]. Subsequently, the Joint Commission on Biochemical Nomenclatures established that the term *glycation*, which until then had indicated the reaction of any carbohydrate with proteins, was to be preferred to *glycosylation*, which meant the addition of glucose alone [8]. Today, by *glycated hemoglobin*, we mean all hemoglobin species deriving from the reaction of any carbohydrate with the terminal valine of the globin chain. However, recent mass spectrometry studies have further demonstrated that glycation, not only of β -globin [9], but also of α -globin, is possible in vivo [10].

As the formation of HbA1c occurs over the average life-span of erythrocytes (about 120 days) and the amount of HbA1c depends on the time-averaged glucose concentration, measurement of the early glycation product of hemoglobin (HbA1c) reflects the extent of exposure to glucose in the 4–8 weeks before testing. This has also been demonstrated in clinical studies, in which HbA1c values have revealed linear correlations between glucose levels in the past 4–8 weeks. After the same period of time, improved glycemic values in diabetic patients with poor metabolic control is reflected in a definite reduction in HbA1c levels [11,12]. In this context, one study which has confirmed the importance of monitoring this parameter in

Table 1

Possible role of AGE in diabetic atherosclerosis



diabetic patients is the Diabetes Control and Complication Trial (DCCT) [13] in which 1441 patients with type 1 diabetes were randomized in two groups, one in intensive insulin therapy (4 injections/day and/or continuous subcutaneous insulin therapy) and intensive glycemic monitoring, and the other with conventional insulin therapy and conventional glycemic monitoring. At the end of the study, the patients in intensive therapy had mean levels of daily plasma glucose of 8.6 mmol/L and HbA1c of 7.2%, significantly lower than those for patients on conventional therapy (mean daily plasma glucose, 12.8 mmol/L; HbA1c, 8.9%). These differences in glycemic control were maintained for a period of 6.5 years and were correlated with a 35–76% reduction of retinopathy, nephropathy, and neuropathy in the group in intensive therapy. At the end of the DCCT study, the patients in the conventional therapy group were offered intensive therapy, and the care of all patients was transferred to their own physicians for evaluation of metabolic control and the occurrence and/or progression of chronic complications annually for 4 years. The results of this study (Epidemiology of Diabetes Intervention and Complications = EDIC) [14], showed that in both groups of patients (previous intensive versus previous conventional therapies), the differences in median HbA1c levels narrowed during follow-up (median over 4 years = 7.9% and 8.2%, respectively). However, interestingly, the DCCT patients on intensive therapy still showed a reduced risk of microangiopathy and in particular, 75% retinopathy, 56% the need for laser therapy, 53% microalbuminuria, and 86% macroalbuminuria, with respect to patients on conventional therapy. These results were also reconfirmed eight years later, as officially noted at the 63rd conference of the ADA: HbA1c levels being equal (8%), patients previously on intensive therapy still showed a reduced risk of microangiopathic complications in particular, 83% the appearance of macroalbuminuria, and 63% progression of retinopathy.

Good glycemic control maintained over time also plays a role in the evolution of macroangiopathic complications, as again shown by the EDIC study [15]. At years 1 and 6 of the study, patients (611 on previous conventional therapy; 618 on previous intensive therapy) underwent B-mode ultrasonography of the internal and common carotid arteries to evaluate intima-media thickness. Results showed that the mean progression of this parameter was significantly less ($P < 0.01$) in the group on intensive therapy during the DCCT than those on conventional treatment (0.032 vs. 0.046 mm); this level of significance was maintained even after adjustment for other risk factors for macroangiopathy such as age, baseline systolic blood pressure, smoking, ratio of low-density to high-density lipoprotein cholesterol, and urinary albumin excretion rate. Thus, intensive therapy during DCCT led to a reduction in the progression of intima-media thickness 6 years after the end of the trial. The apparent effect of diabetes on macroangiopathy may be explained by the gradual accumulation of AGE on vessels [1].

On one hand, these studies clearly highlight the importance of AGE accumulation on the development of long-term diabetic complications; on the other hand, they establish the fact that HbA1c is the “gold standard” in monitoring diabetic patients and defines the “targets” of glycemic control related to the reduction in chronic complications.

Recently, however, in patients with type 1 diabetes, some authors (e.g., [16]) have reported individual differences in the relations between HbA1c levels and the values of mean daily plasma glucose during the 4 weeks before testing: 29% of patients examined showed HbA1c levels which were significantly higher and/or lower than those expected on the basis of plasma glucose values, suggesting the presence of phenotypes with low and high glycation ability. These results open the field to a series of considerations: are there really phenotypes which confer different susceptibility on glycation, an event which may also explain the different susceptibility of diabetic patients to the development of chronic complications? Alternatively, do the techniques currently used for HbA1c measurement also measure other variables, such as the products of glyco-oxidation which have different kinetics with respect to glycation products, the Schiff base in particular, as recently shown [17]? Lastly, the precision of the methods used to dose HbA1c and glycemia is of great importance [12].

There are several methods used to measure HbA1c. Clinicians mainly adopt those which separate the glycated from the unglycated fraction according to the difference in ion charge (ion exchange chromatography by HPLC) or structure (affinity chromatography), colorimetric methods and immunoassay [7,11]. The latter technique uses monoclonal antibodies which specifically recognize glycated amino acids of the β -chain of HbA1c (Table 3) [18].

Table 3
Methods of dosing HbA1c

| Techniques | Advantages | Disadvantages |
|-------------------------------------|------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|
| Ion-exchange chromatography | Can be automated (HPLC) Rapid Reproducible Currently most frequently used | Interferences by abnormal Hb • HbF, HbH:> • HbS, HbC:HbD,HbE:< Need for thermoregulation |
| Commercially available microcolumns | Rapid | Sensitive to variations in temperature Interference of hyperlipemia Rapid variations due to labile fraction Not specific for HbA1c |
| Iso-electrofocusing | Specific for HbA1c No interference by labile fraction or abnormal Hb | Expensive, sophisticated equipment |
| Immunoassay | Specific for HbA1c Recently standardized | High cost Lower values with respect to HPLC |

However, it must be borne in mind that, although measurement of HbA1c levels is now performed in almost all analytical laboratories, it is very delicate to carry out and several precautions must be taken. It is important to remember that, with any method, there are factors which may interfere with measurement, such as the presence of abnormal hemoglobins, carbamylation, acetylation of hemoglobin, or serious hyperlipemia [7,11,19] (Table 3). Furthermore, since the labile and reversible fraction of the Schiff base, which is highly responsive to acute changes in glucose levels, co-elutes with GHb, it may falsely elevate test results and must therefore be removed before assay [7,11]. As regards the accuracy of the determination, the National Diabetes Data Group recommend [7] a target for within- and between-batch coefficient of variations (CV) of 5% for HbA1c, and most methods introduced in clinical practice in the last few years, if used correctly, yield this result [7]. Unfortunately, the rapid spread of these methods and the absence of correct standardization have determined problems in data reproducibility and reliability. Also, techniques which apply the same method often give varying results due to different instrumentation and the lack of a recognized standard [12,20]. Therefore, in 1993, the American Association for Clinical Chemistry [21] formed a committee devoted to standardization of this assay. Due to the lack of an available international reference material and based upon the DCCT data in which HPLC-ion exchange chromatography was used to demonstrate the relationship between levels of glycemia, HbA1c, and the development of chronic complications, the committee recommended that this method serves as a reference method. It must be emphasized that the DCCT method did show good stability and low variability (coefficient of variation, 1.2%). More recently, the International Federation of Clinical Chemistry (IFCC) organized a working group on HbA1c, with the aim of achieving an international system of standardization, developing, and validating a reference method able to measure HbA1c with high specificity, in order to produce a series of control materials, certified, of human origin, to serve as secondary materials of reference and to establish new reference and decision limits. This group has constructed a reference standard for HbA1c which has been quantified by mass spectrometry; on this specific standard, secondary standards have been constructed for use as calibrators for routine HbA1c measurement [22–25]. The procedure has been accepted by all international Society confederates in the IFCC [26]. Unfortunately, due to its high specificity, measuring HbA1c using the IFCC reference standard results in HbA1c values which are 1.5–2.0% lower than those obtained following DCCT standardization. However, a master equation can be applied to convert the results of one reference system to the other [26]. So, once its long-term stability has been proven, the IFCC reference method will be used in all countries. While waiting to be able to use this specific procedure of standardization, it is to be hoped that all laboratories will take into consideration

some fundamental points: the coefficient of variation of the method must be lower than 3%; analytical results must be expressed as HbA1c %; methods used for measurement must be calibrated with calibration materials possessing titres assigned by reference laboratories; and quality control must be executed systematically by all laboratories. For these reasons, it is useful for laboratories to participate in Programs of External Appraisal of Quality, which consist of examining calibrators distributed by the organizers of such programs, and of transmitting their results for verification of the reliability of measurement [26].

HbA1c measurement is therefore a precious parameter for evaluation of glycemic control in diabetic patients, but only if its measurement is reliable and standardized. In this context, it should be applied to all diabetic patients, both at the beginning of the disease, in order to evaluate the degree of metabolic control and, subsequently, as part of continuing care, in order to estimate the effectiveness of the therapeutic approach. For diabetic patients, the American Diabetes Association [27] recommends HbA1c values lower than 7%; in patients who have not reached this target and who have HbA1c levels higher than 8%, the therapeutic program must be carefully re-assessed and, if necessary, modified.

The American Diabetes Association also suggests that the frequency of HbA1c measurements must be established on the basis of patient characteristics: at least twice a year in patients with good metabolic control, and at least every 3 months in ones with poor glycemic control, in whom it is necessary to modify therapy. Proper interpretation of HbA1c values requires that health care providers know the relationship between levels of glycemia and HbA1c, as evidenced by the DCCT study (Table 4), but these values should be used with caution if the HbA1c assay is not standardized and not certified as following the DCCT reference method precisely.

Glycated serum proteins

Glycated serum proteins are the products of the non-enzymatic reaction between serum glucose and the free amino groups of proteins in serum; measurement of the resulting early glycation product expresses metabolic control during the 2 weeks before testing because that is the half-life of albumin and other proteins [11,28]. Among the various methods proposed for measurement of glycated

Table 4
Correlation between HbA1c level and mean plasma glucose levels

| HbA1c (%) | Mean plasma glucose | |
|-----------|---------------------|--------|
| | mg/dl | mmol/L |
| 6 | 135 | 7.5 |
| 7 | 170 | 9.5 |
| 8 | 205 | 11.5 |
| 9 | 240 | 13.5 |
| 10 | 275 | 15.5 |
| 11 | 310 | 17.5 |
| 12 | 345 | 19.5 |

serum proteins, clinicians generally prefer to measure fructosamine [11,28], applying a colorimetric method based on the ability of ketoamine linkages to reduce the dye nitro blue tetrazolium (NBT) and to produce a compound which absorbs at 525 nm. Unfortunately, reaction with NBT is not specific for ketoamine linkages, since other reducing agents in serum, such as ascorbic acid, uric acid, and glutathione, can interfere with measurements. Other substances which absorb at 525 nm, like bilirubin and triglycerides, when present in serum at elevated concentrations, can also interfere with measurements and cause artificially elevated fructosamine values. As albumin is the most abundant protein in serum and contains multiple lysine residues, measurement of fructosamine is mainly determination of glycated albumin, and variations in its concentration in serum may give fructosamine values which do not correlate with glycemic levels. In fact, there is continuing debate as to whether the fructosamine assay should be corrected for serum albumin or serum protein concentrations. So, in view of the limitations of fructosamine measurement, this parameter should only be used in particular situations: for example, when indications of short-term changes in glycemic control are needed [29,30], in cases of major changes in therapy or diabetic pregnancy, or when there are factors which alter red blood cell survival and influence interpretation of HbA1c levels (e.g., hemoglobinopathies, hemolytic anemia) [27].

Measurement of intermediate glycation products

Intermediate glycated products which can currently be estimated are glyoxal, methyl glyoxal and deoxyglucosones. Some studies have shown that in diabetic rats, methyl glyoxal formation is high in kidneys, lens, and plasma, and that it increases proportionally to glucose concentration [31]. Methyl glyoxal can form cross-links and modify some enzymes of the glycolysis pathway, microtubular proteins, and collagen [31]. Furthermore, in vivo studies have demonstrated that methyl glyoxal levels are five to six times higher in type 1 diabetics and two to three times higher in type 2 diabetic patients, with respect to controls. In one of our recent studies, in type 2 diabetic patients with bad metabolic control, attaining good control with optimized insulin therapy was not successful in normalizing either glyoxal or methyl glyoxal levels after 6 months, unlike HbA1c and fructosamine values, thus emphasizing that these products, like AGE, need more stable, prolonged, good metabolic control for their normalization [32]. There are several methods for measuring glyoxal and methyl glyoxal, the most specific being HPLC and gas chromatography/mass spectrometry (GC/MS) [33,34].

High levels of 3-deoxyglucosone have been found in the plasma of diabetic subjects [4,28]. This compound, in vitro, is able to cause cross-links in proteins, so its serum and tissue levels may be correlated with chronic diabetic complications.

In this context, higher levels of 3-deoxyglucosone have recently been reported in patients suffering from more severe microangiopathic complications (nephropathy and retinopathy) [35]. The elevated reactivity of this compound means that its measurement by HPLC is difficult [28].

Measurement of advanced glycation end-products (AGE)

Due to the chemical characteristics of advanced glycation end-products, they were initially measured by spectroscopic and fluorimetric methods, exploiting their particular values of absorption (280 nm) and fluorescence (emission at 440 nm, excitation at 370 nm). However, these methods are not very specific and can only give indications on the general trend of the glycation process [1]. More recently, RIA and ELISA methods have been developed, using polyclonal antibodies raised against AGE and obtained in vitro from the glycation reaction of glucose with ribonuclease. These antibodies react with the AGE forming in vivo and, by these methods, high AGE levels have been found in the plasma and tissues of diabetic subjects [28]. However, AGE are estimated totally, and reactivity with other substances similar to them cannot be excluded [36].

The structural characterization of AGE is particularly difficult due to their chemical complexity, and this means that the identification of some compounds, including FFI [37], was subsequently demonstrated to be artifacts caused by hydrolysis and the neutralization procedure [38,39].

Two AGE, pentosidine and carboxymethyl lysine, have recently been structurally characterized. They are considered glyco-oxidation products because pro-oxidizing conditions are necessary for their formation [3,4,28]. Pentosidine is a cross-link with characteristic fluorescence which derives from the reaction of glucose with lysine, arginine and ribose, with the formation of an imidazopyridine ring; this compound also forms from the reaction of arginine and lysine with glucose, fructose and other reducing sugars. In plasma, approximately 95% of pentosidine is linked to proteins, 5% is present in its free form. Free pentosidine, due to its very low concentrations, cannot be detected in either normal or diabetic subjects with normal renal function; accumulation of free pentosidine is, in fact, determined by decreased glomerular filtration [40].

Isolated initially in the collagen of diabetic subjects, high levels of pentosidine were then found in plasma, red cells, and urine [41]. Pentosidine levels are also correlated with age and the duration of diabetes; its levels are higher in diabetic patients with micro- and macro-angiopathic complications, and linearly related to their severity [42]. Although it is now possible to measure pentosidine levels accurately with an HPLC method [28,41], unfortunately, a pentosidine standard is still not available commercially, and this makes its measurement only applicable for research purposes.

Carboxymethyl lysine (CML) is a glyco-oxidation product, not reactive and not fluorescent, which derives from oxidative degradation of Amadori products [28]. In collagen and lens, its levels increase linearly with age. High levels of CML have been shown in the collagen of diabetic patients and are linked with retinopathy and nephropathy [28]. Recent studies also indicate that peroxidation of lipids, together with glycation, may be a source of CML in diabetes and atherosclerosis [43]. Initially estimated by GC/MS, carboxymethyl lysine [28] may now be measured with an ELISA technique which uses monoclonal antibodies; unfortunately, this method is not yet on the market, so that measurement of this parameter can only be used for research [28].

A third product of advanced glycation is pyrraline, first isolated *in vitro* and later found *in vivo*, although in low concentrations. Its elevated reactivity explains the difficulty in quantifying it accurately in biological materials [28]. The presence of this compound in glomerular basement membrane and collagen has been verified by an ELISA technique using mono- and polyclonal antibodies. Higher levels of pyrraline have been found in the urine of diabetics than in control subjects by an HPLC method [28].

AGE in renal failure

It has recently been demonstrated that AGE, pentosidine and CML levels are higher in subjects affected by renal failure, diabetic or not, with respect to those of diabetic patients with normal renal function [40,44,45]. In the serum of subjects affected by renal failure, pentosidine can also be measured in its free form [40]. Although pentosidine is mainly derived from the reaction of proteins with glucose and fructose, it has been shown that, in patients with end-stage renal disease, it correlates with the plasma levels of ascorbate and the oxidized form of ascorbate, suggesting that dehydroascorbate is an important precursor of pentosidine in

this disease [46]. Instead, CML may be formed by the auto-oxidation of amino acids, carbohydrates, lipids, and products of lipid peroxidation [46]. Furthermore, recently, proteins modified with malondialdehyde, a product deriving from the oxidation of polyunsaturated fatty acids such as arachidonate, and by other lipids called Advanced Lipoxidation Products (ALE) have been isolated in the plasma of uremic patients [47]. The accumulation of AGE and ALE in uremia is not related to glucose, fructose-lysine or triglyceride levels [48,49], suggesting that other factors are important in determining the formation of these compounds [50]. In addition, 90% of pentosidine and CML are linked to proteins in the plasma of uremic patients, so decreased renal clearance of protein-linked AGE and CML cannot be invoked [48,49].

Recent evidence strongly suggests that in uremia, increased concentrations of small reactive carbonyl compounds (RCO), derived from carbohydrates, lipids and amino acids, such as glyoxal, methyl glyoxal, 3-deoxyglucosone, glyceraldehyde, dehydroascorbate and malondialdehyde give rise to AGE and ALE. So, in uremia, the increased formation of carbonyl compounds and the subsequent carbonyl modification of proteins cause a situation of “carbonyl stress” [47] (Fig. 5), which has been implicated as a pathogenic factor in a series of complications characteristic of this disease. The increased carbonyl stress in uremia may be due both to increased formation of RCOs by oxidative stress [47] or their decreased detoxification and clearance [50].

Among uremic complications, dialysis-related amyloidosis, accelerated atherosclerosis, and dialysis-related bone lesions can be exacerbated by carbonyl stress.

Dialysis-related amyloidosis causes large-scale joint destruction and is caused by amyloids, mainly due to β_2 microglobulin deposition in joints [51]. It has recently been shown that glycation of β_2 microglobulin occurs *in vivo* [52], suggesting that AGE- β_2 microglobulin is able to induce chemotaxis of monocytes, synthesis of interleukines 1b and

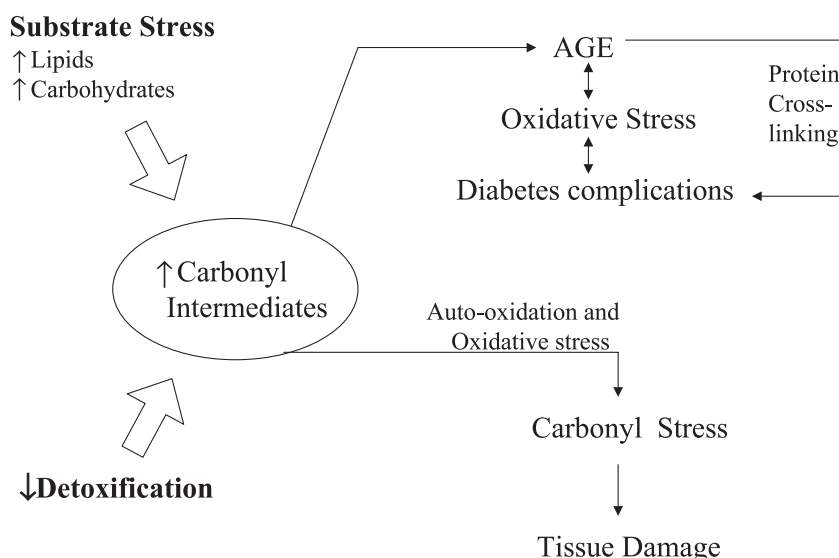


Fig. 5. Carbonyl intermediates and their fate in diabetes or uraemia.

6, and production of collagenase in synovial cells, thus determining inflammation and erosive arthropathy [52]. Dialysis-related bone lesions may be caused by the stimulation of osteoclast bone absorption due to carbonyl compounds [53].

Proteins modified by AGE and ALE show a series of biological activities, including the capacity to initiate inflammatory responses and to induce the vascular modifications described above, all of which well explain the accelerated atherosclerosis characteristic of uremic patients. In addition, some inflammatory parameters such as fibrinogen, orosomucoid, and pregnancy-associated protein A, have recently been found to be higher in chronic hemodialysis patients than in normal subjects and related to RCO products [54]. This assumption is confirmed by studies on normal subjects (e.g., [55]), showing that AGE levels are higher in arterial tissue.

Reactive carbonyl accumulation leading to carbonyl stress has been invoked as a cause of the glomerular damage observed in subjects with diabetes and end-stage renal disease [45]. Increased serum levels of AGE may result in deposition of AGE in the mesangium, Bowman's capsule, and capillary loops leading to progressive renal damage through increased glomerular volume, mesangial expansion, basement membrane thickening, proteinuria and glomerulosclerosis [56].

It has recently been shown that AGE receptors deplete to bind and degrade AGE-modified proteins release small molecular peptides. In normal subjects, these AGE-peptides are filtered and reabsorbed by the proximal tubules and metabolized [57,58]. Impairment of renal function determines a progressive increase in AGE-peptides, as has been shown in the plasma of diabetic and non-diabetic patients with end-stage renal disease [59]. These peptides are highly reactive and may react with plasma lipoproteins to form AGE-modified LDL and cross-links to collagen. Thus, AGE

peptides exhibit the same toxic activity as AGE and this may explain both the further progression of chronic complications in diabetic patients with renal disease and the accelerated atherosclerosis that occurs in patients with end-stage renal disease [60,61].

Clinical data on AGE-peptides have yielded a series of AGE products isolated from plasma by ultrafiltration at a molecular mass cut-off of 10,000 kDa [58,61]. So, considering the importance of structural characterization in understanding the pathophysiological implications of these substances in diabetes and end-stage renal disease, we undertook a series of investigations on in vitro glycated human serum albumin followed by enzymatic digestion, applying various mass spectrometry approaches (liquid chromatography/electrospray ionization mass spectrometry [ESI/MS], matrix-assisted laser desorption mass spectrometry [MALDI/MS]) for structural information on these peptides. These approaches yielded a series of glycated peptides which were identified in in vitro conditions [62,63]; studies are now in progress to verify their possible presence in vivo.

In this context, dialysis techniques play an important role in AGE clearance, and a series of studies have shown that current methods of renal replacement therapy like hemodialysis (HD) and peritoneal dialysis (CAPD) are only partially effective in reducing serum AGE in patients with end-stage renal disease, whether they are diabetic or not [64–69]. In HD patients, about 50% of plasma AGE peptides can be removed by high-flux dialysis, since low-flux dialysis is less efficient [70,71]. Hemodialysis has little effect in reducing total AGEs [72], plasma protein bound pentosidine and CML [67], whereas the levels of free pentosidine are reduced by about 80% with low-flux as well as high-flux dialysis [68]. As for CAPD, some authors have reported lower levels of plasma pentosidine in patients on CAPD with respect to those on HD [73]; others report no differences between the two methods [74].

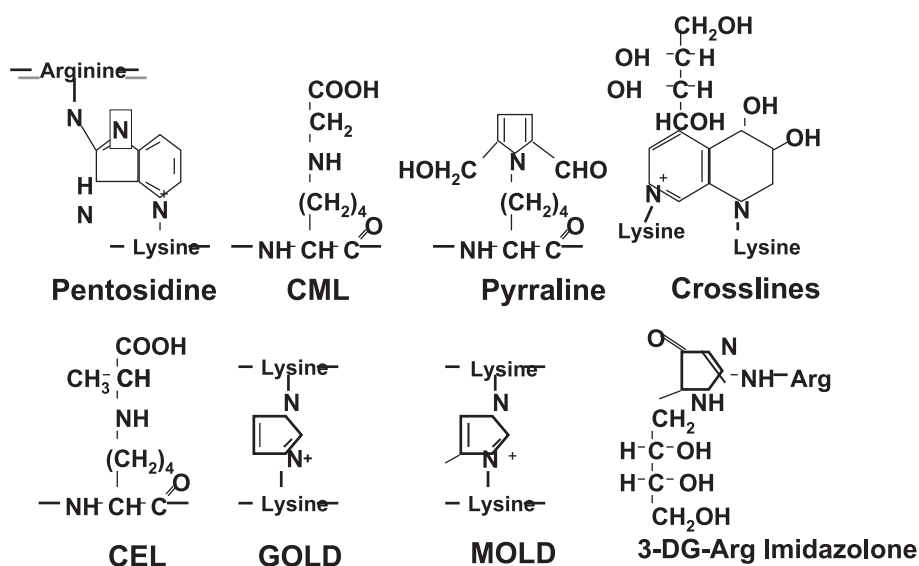


Fig. 6. Identified and recently described AGE.

In this context, it should be emphasized that the loss of ultrafiltration capacity of the peritoneal membrane is a complication often observed in patients who have been on peritoneal dialysis for a long time [75]. This alteration is associated with increased permeability to small molecular-weight solutes. Some studies have revealed that, in non-diabetic CAPD patients, progressive accumulation of RCO, AGE, and ALE on the peritoneal membrane occurs and that the severity of the changes is related to the extent of AGE accumulation [76–78]. In fact, it has been showed that these compounds can determine the peritoneal membrane dysfunction, both by modifying peritoneal matrix proteins and altering their structure, and also by reacting with endothelial peritoneal cells and inducing an inflammatory response [79]. It has also been demonstrated that heat sterilization of glucose-based peritoneal dialysis solutions leads to the formation of RCO [80].

Renal transplant is certainly the best therapeutic tool in terms of AGE reduction, as has been shown in a series of studies [70,81,82] showing normalization of AGE-peptide and free pentosidine levels within days to weeks after kidney transplant. Serum levels of protein-bound pentosidine and CML were reduced but not normalized after 6 months from operation [81]. These data are further supported by those obtained on tissue AGE levels: tissue pentosidine levels are still high 6 months after transplant [81]: so a longer period of normal renal function is required to allow these compounds to normalize—a fact which may contribute to transplant complications.

Thus, evaluation of AGE and particularly of pentosidine levels constitutes a physiopathological marker useful in estimating AGE distribution, metabolism and turnover in selected patients. It is also a prognostic marker of the chronic complications of diabetes and end-stage kidney disease, and a therapeutic marker for evaluating the effectiveness of AGE inhibitors.

However, from all the above, it is also clear that the complexity of the glycation reaction and the high numbers of resulting compounds make structural characterization difficult, although new and highly specific methods, such as mass spectrometry, have made and will continue to make important contributions in this context [83]. Recently, in fact, these approaches have meant that a series of possible AGE compounds have been isolated and characterized in vitro [84,85], and in vivo studies in the near future will provide indications on their possible use for clinical purposes (Fig. 6).

Conclusions

Non-enzymatic protein glycation plays a role in the evaluation of metabolic control, and in our understanding of the pathogenetic mechanisms of chronic complications and the relationship between metabolic equilibrium and the development of complications in diabetes.

Measurement of *early glycation products* has shown itself to be a precious and consolidated instrument in the metabolic monitoring of diabetic patients.

The possibility of being able to monitor some *intermediate* and *advanced glycation end-products* will certainly provide important information on the pathogenesis and progression of chronic complications, and will be useful in verifying the effectiveness of anti-oxidants and glycating substances in diabetes. Their monitoring in end-stage kidney disease will also make a precious contribution not only to our understanding of how “carbonyl stress” causes the chronic damage characteristic of this condition, but also on how to define the most effective therapeutic approaches toward reducing it. It is therefore to be hoped that the methodological problems in measuring these compounds will soon be solved, so that they can be used to monitor diabetic patients effectively.

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