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Diabetic threesome (hyperglycaemia, renal function and nutrition) and advanced glycation end products: evidence for the multiple-hit agent?

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Complex chemical processes termed non-enzymic glycation that operate *in vivo* and similar chemical interactions between sugars and proteins that occur during thermal processing of food (known as the Maillard reaction) are one of the interesting examples of a potentially-harmful interaction between nutrition and disease. Non-enzymic glycation comprises a series of reactions between sugars, α-oxoaldehydes and other sugar derivatives and amino groups of amino acids, peptides and proteins leading to the formation of heterogeneous moieties collectively termed advanced glycation end products (AGE). AGE possess a wide range of chemical and biological properties and play a role in diabetes-related pathology as well as in several other diseases. Diabetes is, nevertheless, of particular interest for several reasons: (1) chronic hyperglycaemia provides the substrates for extracellular glycation as well as intracellular glycation; (2) hyperglycaemia-induced oxidative stress accelerates AGE formation in the process of glycoxidation; (3) AGE-modified proteins are subject to rapid intracellular proteolytic degradation releasing free AGE adducts into the circulation where they can bind to several pro-inflammatory receptors, especially receptor of AGE; (4) kidneys, which are principally involved in the excretion of free AGE adducts, might be damaged by diabetic nephropathy, which further enhances AGE toxicity because of diminished AGE clearance. Increased dietary intake of AGE in highly-processed foods may represent an additional exogenous metabolic burden in addition to AGE already present endogenously in subjects with diabetes. Finally, inter-individual genetic and functional variability in genes encoding enzymes and receptors involved in either the formation or the degradation of AGE could have important pathogenic, nutrigenomic and nutrigenetic consequences.

Advanced glycation end products: Diabetes mellitus: Diabetic nephropathy: Nutrigenetics

Diabetes mellitus is the most common metabolic disease. Its prevalence is steadily rising (with few exceptions) globally, having already reached epidemic proportions in industrialised countries. Current WHO estimates predict that the number of individuals with diabetes will reach approximately 300 million by 2025[1]. The diabetes mellitus epidemic is paralleled by body-weight changes (overweight or obesity), and in both cases there is a clear-cut correlation; the faster the socio-economic progress (and subsequent lifestyle changes) in a given society the steeper the rise in the incidence and prevalence of diabetes mellitus and obesity. Both common types of diabetes mellitus, type 1 (T1DM) and type 2 (T2DM), are ethiopathogenetically 'complex' diseases with a certain extent of genetic predisposition and a substantial influence of environmental factors (particularly diet and physical (in)activity), although the phenotypic complexity and prevalence of T2DM is much higher. Since diabetes mellitus is characterised by profound abnormalities in energy-substrate partitioning as a result of deficient insulin action (either absolute or relative), nutrition plays a particularly important role in disease progression and its long-term outcomes.

Energy-substrate overload in diabetes (i.e. hyperglycaemia and high NEFA) leads to multiple alterations of intermediate metabolism, presumably as a result of hyperglycaemia- and NEFA-driven overproduction of reactive oxygen species (ROS) in the mitochondrial

Abbreviations: AGE, advanced glycation end products; CML, N_ε-carboxymethyl-lysine; CRP, C-reactive protein, FN3K, fructosamine-3-kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RAGE, receptor for AGE; ROS, reactive oxygen species; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

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respiratory chain (since both glucose and NEFA provide the same electron donors NADH and $FADH_2$)[2,3]. One of these alterations leads to the formation of heterogeneous moieties, collectively termed advanced glycation end products (AGE), by the processes of non-enzymic glycation and glycoxidation, which comprise a series of reactions between reducing sugars, α-oxoaldehydes and other sugar derivatives and amino groups of amino acids, peptides and proteins. A mounting body of evidence suggests that AGE possess a wide range of chemical and biological effects and play a role in diabetes-related pathology as well as in several other diseases[4–7]. Similar chemical interactions between sugars and proteins occur during thermal processing of food and have been known to the food chemists for approximately 100 years as a result of the pioneering studies of Louis Camille Maillard[8]. This chemical interaction is termed the Maillard reaction and the compounds formed are Maillard reaction products; chemically they are largely similar to the AGE formed *in vivo*. Diet is believed to be a potentially important source of exogenous AGE; however, assessment of the true impact of dietary AGE has recently been the subject of intensive research.

The current and in particular the future extent of the diabetes mellitus epidemic makes it crucial to understand its ethiopathogenesis (including the role of AGE) from the whole body to the molecular level. The following review summarises: (A) the contribution of the principal pathogenic features of diabetes (chronic hyperglycaemia) to the enhanced formation of endogenous AGE, and the biological effects of AGE; therefore establishing the 'first' hit of the multiple-hit hypothesis being put forward in the present paper for the role of AGE in diabetes; (B) the current knowledge of the metabolic fate of AGE, the renal excretion of AGE and its impairment by diabetic nephropathy (as well as other causes), which might thus constitute the 'second' hit in increasing the AGE burden; (C) the most controversial topic, and potentially the 'third' hit, i.e. whether increased dietary intake of AGE in highly-processed foods consumed by patients with diabetes might represent an important source of AGE for the organism in question and, therefore, pose the additional metabolic burden (the 'third' hit); (D) in addition, based on data available, the possibility that an individual's genetic make-up may influence the formation and processing of AGE (i.e. nutrigenetics) and that AGE, conversely, may influence the integrity and expression of the genome (i.e. nutrigenomics).

The 'first' hit: increased formation of endogenous advanced glycation end products

Metabolic derangements in diabetes and mechanisms of cell damage by hyperglycaemia

Absolute (T1DM) or relative (T2DM) insulin deficiency results in impaired postprandial glucose uptake by skeletal muscle, decreased glucose processing into glycogen, inadequate suppression of hepatic gluconeogenesis and lipolysis in adipose tissue (NEFA further interferes with glucose oxidation via the glucose–NEFA cycle). If the cell cannot efficiently down regulate insulin-independent glucose intake in hyperglycaemia it becomes overloaded, which has been convincingly shown for endothelial[9,10], mesangial[11] and renal tubular[12] cells, all of which are targets for vascular damage by hyperglycaemia in diabetes. In the case of endothelial cells the expression of some types of facilitative glucose transporters stay stable or are moderately down regulated by glucose (GLUT1), while others (GLUT2) are up regulated[9,10]. Intracellularly, glucose is metabolised via glycolysis and the Krebs cycle, and the electron donors (NADH and $FADH_2$) generated then supply electrons for the electron transport chain in the mitochondria to be used to generate H^+ membrane potential for the production of ATP via ATP synthase. Increased availability of glucose and glycolytic intermediates have been shown to provide substrates for several pathways that are believed to be largely responsible for the cell damage induced by hyperglycaemia[2]: polyol and hexosamine pathways; dicarbonyl production (mainly methylglyoxal by degradation of triosephosphates) and non-enzymic glycation (AGE); *de novo* synthesis of diacylglycerol with subsequent activation of protein kinase C isoforms. The overall effects of these abnormalities are changes in gene expression by activation of certain transcription factors (mainly NF-κB), post-translational modification of intra- and extracellular proteins (glycation), alteration of cellular signalling (by activation of kinases), endoplasmic reticulum stress leading to unfolded protein response, impairment of antioxidant capacity etc. (for details, see Brownlee[2,13]).

Recently, a 'unifying' hypothesis was proposed that strengthens the role of mitochondrial ROS overproduction and subsequent DNA oxidative damage as a potent accelerator of these abnormalities (originally seen merely as a result of increased substrate availability because of allosteric inhibition and thus accumulation of glycolytic intermediates). Hyperglycaemia overloads the mitochondrial electron transport chain by increased availability of reducing equivalents (mainly on complex III) and a larger proportion of O_2 is then partly reduced with single electrons to superoxide[13]. Overexpression of manganese superoxide dismutase or uncoupling protein-1 abolishes hyperglycaemia-induced ROS generation *in vitro*[14]. In fact, $NADH:NAD^+$ rather than the absolute amount of NADH seems to be important for the intensity of mitochondrial superoxide generation[15], and since hyperglycaemia-induced ROS generation directly contributes to an increase in the $NADH:NAD^+$ (see later), diabetes represents a vicious circle in relation to oxidative stress. ROS can oxidatively damage most types of macromolecules; however, oxidative damage of DNA (strand breaks) is particularly important since it requires energy-consuming repair. Ribosylation of multiple proteins by poly-ADP-ribose polymerase, including auto-ribosylation of poly-ADP-ribose polymerase, itself initiates and facilitates DNA repair[16]. NAD^+ serves as a donor of ADP-ribose and its consumption by poly-ADP-ribose polymerase contributes to its decreased availability (an increase in $NADH:NAD^+$), further ROS generation and a decrease in ATP formation. Pharmacological inhibition of poly-ADP-ribose polymerase *in vitro* can suppress all these abnormalities in streptozotocin-induced diabetes[17].

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Depletion of NAD^+ also inhibits the key glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and causes the accumulation of upstream glycolytic intermediates and their processing in alternative metabolic pathways (see earlier discussion, p. 61). Experimentally, GAPDH antisense oligonucleotides applied to cells cultured in 5 mM-glucose completely mimics hyperglycaemia by activating pathways leading to vascular damage to the same extent as in 30 mM-glucose culture conditions[18]. GAPDH is generally viewed as a classical glycolytic enzyme; however, it is now clear that it is a multifunctional protein with diverse functions in numerous cellular processes including apoptosis and DNA repair[19–21]. GAPDH translocates to the nucleus under a variety of stressors, most of which are associated with oxidative stress. Interestingly, poly-ADP-ribosylation of GAPDH itself seems to be one of the likely events enabling its nuclear translocation (further slowing down the rate of glycolysis) and yet another factor responsible for the harmful effect of hyperglycaemia[18].

Formation of advanced glycation end products in vivo *by non-enzymic glycation*

Non-enzymic glycation (also termed the Maillard reaction in some contexts) of proteins involves interplay of serial and parallel reactions between protein residues (mainly lysine and arginine) and sugars, α-oxoaldehydes (dicarbonyls) and other sugar derivatives (*in vivo* mainly triosephosphate derivatives) generating early (pre-AGE) and advanced glycation products (AGE)[22]. Glycation is one of the most common types of protein modification and spontaneous damage of proteins, which affects approximately 0·1–0·2% of the arginine and lysine residues *in vivo*[22]. Glycation of proteins proceeds with variable rate and extent during the lifespan of proteins in tissues and body fluids under physiological conditions, but it is more intensive in several disease conditions such as diabetes[23], atherosclerosis[24], neurodegenerative diseases[25], osteoarthritis[26] and renal failure[27]. The rate of reaction depends on variables such as temperature, pH and carbonyl:amine, and is thus topically and temporally variable. Generation of some AGE is substantially enhanced in the presence of ROS and some AGE are formed exclusively under oxidative conditions (hence the alternative term 'glycoxidation'). Originally, glycation was considered to be a type of post-translational modification that occurs mostly in long-lived proteins (e.g. collagen, crystalline); however, it is now clear that AGE are formed also in short-lived proteins, including the cellular proteome[4,22].

Glycating agents *in vivo* include free sugars (glucose), glycolytic intermediates such as sugar phosphates (glucose- and fructose-6-phosphates) and dicarbonyls (methylglyoxal, glyoxal and 3-deoxyglucosone). Sugars are reactive towards lysine residues while dicarbonyls are mainly reactive towards arginine residues of proteins. Glycation by sugars proceeds through the early (Schiff's bases) and intermediate stages (Schiff's bases undergo the Amadori rearrangement to fructosamines) towards the formation of heterogeneous moieties collectively termed AGE. If the initial glycating agent is glucose the initial product is termed a fructosamine. Probably the best-studied fructosamine to date is HbA_{1c}, which is glycated on the NH_2-terminal valine of the β-globin chain, although Hb can also be glycated on several other lysine residues. Since its discovery in 1968[28] HbA_{1c} has been established as a widely used and very useful marker of medium- to long-term compensation of diabetes with a certain predictive value[29]. Quantitatively, however, fructoselysine is the most abundant early glycation product and fructosamine *in vivo*. Fructosamines are unstable products that slowly degrade to form AGE.

Fructosamines were traditionally considered to be a major source of AGE *in vivo*. Recently, however, as a result of advances in the methodology available for the precise chemical characterisation and quantification of pre-AGE and AGE (particularly liquid chromatography with tandem MS[30]), it has became apparent that dicarbonyl-derived AGE are the predominant class of AGE *in vivo*[22]. Dicarbonyls are formed from the triosephosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (methylglyoxal), lipid peroxidation (glyoxal), fragmentation of early glycation products by the Namiki pathway[31] (glyoxal, 3-deoxyglucosone), oxidative degradation of nucleotides (glyoxal) and ketone body metabolism (methylglyoxal) and by enzymic degradation of fructosamines[32,33] (3-deoxyglucosone). In diabetes the triosephosphates are the most important source of dicarbonyls as a result of the previously described inhibitory effects of hyperglycaemia on GAPDH function. GAPDH activity has been shown to be the most important determinant of methylglyoxal concentration for a given glucose level (with an inverse correlation between GAPDH activity and methylglyoxal level)[34]. The products of sugar oxidation, methylglyoxal, glyoxal and 3-deoxyglucosone, react with mainly arginine residues of proteins and form AGE directly. Dicarbonyl-derived AGE on arginine residues, the hydroimidazolones (methylglyoxal-H, glyoxal-H, 3-deoxyglucosone-H), are the most abundant AGE *in vivo* in body fluids as well as in cellular protein (representing approximately 1% of the total arginine in some cell types)[22]. The concentration of dicarbonyls as well as dicarbonyl-derived hydroimidazolones has been shown to be significantly increased in patients with diabetes (≤3-fold for glycated plasma proteins, and ≤10-fold for glycation-free adducts; see p. 65) compared with controls without diabetes[35,36]. The level of dicarbonyls correlates with glycaemia, and variations in postprandial glucose contribute substantially to the rise of dicarbonyls (and thus dicarbonyl-derived AGE), while levels of HbA_{1c} do not reflect the postprandial glucose fluctuations[37]. Apart from the arginine-based dicarbonyl-derived AGE several other classes of AGE have been identified and chemically characterised (an overview of the current AGE classification based on their chemical structure and source is presented in Table 1).

Enzymic defence against glycation

Although the formation of pre-AGE and AGE is a non-enzymic process, e.g. methylglyoxal arises by non-enzymic phosphate elimination from the two intermediates

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Table 1. Current classification of advanced glycation end products (AGE)

Class	Members	Source and target AA	Relative quantity *in vivo*
Hydroimidazolones (HY)	G-HY MG-HY 3DG-HY	G, Arg MG, Arg 3DG, Arg	Absolutely highest *in vivo* (approximately 90% of all glycated plasma protein) with MG-HY predominance
Monolysyl adducts	N_ε-carboxymethyl-lysine N_ε-carboxyethyl-lysine Pyrraline	G, Lys MG, Lys 3DG, Lys	10 × lower than HY (approximately 10% of all glycated plasma protein)
Bis(lysyl) imidazolium cross-links	MG-derived lysine dimer (MOLD) G-derived lysine dimer 3DG-derived lysine dimer Glucosepane	MG, Lys G, Lys 3DG, Lys FN, Lys	Minimally (<1% of all glycated plasma protein) with MOLD predominance
AGE fluorophores	Pentosidine Argpyrimidine	3DG, Lys or Arg MG, Arg	100–1000 × lower than HY (approximately 1% of all glycated plasma protein)

3DG, 3-deoxyglucosone; FN, fructosamine; G, glyoxal; MG, methylglyoxal; AA, amino acid.

of glycolysis glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (approximately 0·2–1% of the flux of triosephosphates under normoglycaemic conditions[38]), organisms including man have developed several enzymic detoxification systems to deal with those products[39], since high levels of methylglyoxal, for example, are highly cytotoxic. Surprisingly, the physiological role of dicarbonyls including methylglyoxal is still rather obscure (methylglyoxal is known to target several proteins involved in the regulation of cell growth, differentiation and cell death). From an evolutionary point of view monosaccharides such as glucose have evolved as a universal source of energy and C for metabolism; however, because of their intrinsically-high reactivity with amines, non-enzymic glycation is an unavoidable 'background' reaction in all living systems[40]. Since uncontrolled glycation is detrimental to the function and integrity of biological macromolecules (the production of ROS from early glycation products, impairment of enzyme functions, perturbations of peptide hormone signalling, activation of AGE-specific receptors, cross-linking of structural proteins, impairment of protein recycling, mutagenicity etc.; see later) the need for regulatory systems capable of limiting and reversing such protein modifications has evolved in parallel[40]. Passive regulatory mechanisms to limit unwanted excessive glycation include preference for monosaccharides with the lowest reactivity towards amines (i.e. glucose) and the maintenance of the lowest glycaemia possible or necessary as a result of prompt disposal of excess glucose to storage forms under insulin regulation. However, exclusively active mechanisms (deglycating enzyme systems) can (at least partly) maintain homeostasis when there are physiological or pathophysiological variations in the levels of glycating substrates. One of these detoxification mechanisms is the glyoxalase system[41]. Methylglyoxal reacts spontaneously with glutathione to form a hemithioacetal, which is converted into S-D-lactoylglutathione by glyoxalase I, and then further metabolised to D-lactate by glyoxalase II. The importance of methylglyoxal and its detoxification in hyperglycaemia has been confirmed experimentally; glyoxalase I overexpression *in vitro* completely protects against the hyperglycaemia-driven formation of methylglyoxal and AGE in bovine endothelial cells[42]. Similarly, fructoselysines may be enzymically degraded by fructosamine-3-kinase (FN3K)[32,33]. FN3K phosphorylates fructoselysine residues on glycated proteins yielding fructosamine 3-phosphates, which are unstable and spontaneously decompose to form 3-deoxyglucosone. Based on current knowledge of both these enzymic systems (and several others such as aldehyde reductases and dehydrogenases and amadoriase) it is clear that deglycation plays a regulatory role under the normal metabolic conditions and efficacy of the systems may, consequently, play an important role in hyperglycaemia[43]. Whether the genetic variability in the glyoxalase and FN3K loci influence their efficacy will be discussed later (p. 68).

Biological effects of advanced glycation end products

AGE exert multiple biologically-important effects in living organisms by direct modification of proteins (and their functions) and indirectly via AGE-binding receptors. The importance of direct glycation certainly applies to long-lived proteins such as extracellular matrix proteins (e.g. collagen); their cross-linking (lysine to lysine or lysine to arginine residues), rigidity and resistance to proteolysis contribute to the changes in physical properties observed during aging, with accelerated rates in tissues (e.g. skin collagen) from patients with diabetes[44]. If glycation occurs at specific sites it can also affect the cell–collagen interaction, e.g. modification of arginine within the RGD or GFOGER motifs recognised by integrins $\alpha1\beta2$ and $\alpha2\beta1$, which reduces cell interactions during turnover and platelet interactions and can ultimately affect tissue repair and wound healing in patients with diabetes[45]. Arginine-derived hydroimidazolone modification of integrin-binding sites of vascular-basement-membrane type IV collagen has been shown to induce endothelial cell detachment, anoikis and inhibition of angiogenesis, therefore possibly contributing to vascular damage in diabetes[46]. In proteins with a faster turnover abnormal protein–protein

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or enzyme–substrate interactions can be affected by glycation. The most-abundant plasma protein in man, human serum albumin, is an important target of glycation *in vivo*. Peptide mapping of human serum albumin has identified hot spots for glycation by dicarbonyls (methylglyoxal) located in the drug-binding site and the active site of albumin-associated esterase activity[47]; hot spots for modification by glucose have also been identified [48]. Similar hot spots for dicarbonyl-derived arginine-directed modification (in addition to glucose-derived lysine-directed modification) have been identified in Hb[49], both types of modifications have been shown to contribute to increased susceptibility to auto-oxidation, reduced α-helix content, increased thermolability and a weaker haem–globin linkage in glycated Hb[50]. These examples are just some of the glycated proteins studied; structural modification of many other proteins (such as apo, extracellular matrix proteins, crystalline etc.) also contributes to diabetic pathology. Another interesting effect of AGE has come from an *in vitro* study demonstrating that AGE dose-dependently impairs CD34+ progenitor cell function (i.e. incorporation of progenitor cells into the sprouting endothelium), which might be yet another pathophysiological factor (mediated by a hitherto unknown mechanism) of disturbed vascular function in diabetes[51].

Although most of the research has focused on the effect of AGE on proteins, it has become increasingly obvious that DNA serves as another target for AGE modification. The genotoxicity of AGE has been documented *in vitro* in several cell lines[52,53] and *in vivo* in patients with uraemia who are undergoing different types of haemodialysis[54]. DNA damage is induced by both the production of ROS via receptor-mediated signalling, probably involving the receptor for AGE (RAGE)/NAD(P)H oxidase pathway (estimated by 8-hydroxy-2-deoxyguanosine), and by the direct effect of AGE on DNA producing base modifications, stand breaks, photosensitisation and apurination or apyrimidination.

Circulating AGE-modified molecules also interact with specific cell-surface receptors on a range of cell types (in the circulation predominantly on monocytes and macrophages and endothelium). In some cases AGE undergo endocytosis and degradation, in others they activate intracellular signalling pathways and influence cellular phenotype. Several types of receptors for AGE-modified molecules have been identified: RAGE[55,56]; galectin-3[57]; OST-48; 80K-H[58]; scavenger receptors class A (I and II)[59] and B (CD36 and BI); lectin-like oxidised LDL receptor-1; FEEL-1 and -2; probably others as yet unidentified[60]. RAGE, isolated and characterised in 1992[55,61], is an Ig-type cell-surface receptor that recognises the tertiary structure of multiple ligands including S100/calgranulins[62] and high-mobility-group box-1 protein[63], amyloid β-peptides[64] and AGE. RAGE has similarities with signalling pattern-recognition receptors such as Toll-like or mannose-receptors that are expressed by immune cells and recognise proteins associated with microbial pathogens or cellular stress and participate predominantly in the innate immune response. Accumulating experimental data on RAGE functions and actions have been extensively reviewed throughout the past decade, particularly in the most recent comprehensive reviews[65–68]. Binding of AGE to RAGE has been shown to activate several intracellular signal transduction pathways (probably tissue- and cell-type-specific), mainly mitogen-activated protein kinases including extracellular regulated kinase 1/2, p38 mitogen-activated protein kinase and stress-activated protein kinase/c-Jun N-terminal kinase; furthermore, other pathways identified are the janus kinase/signal transducers and activators of transcription pathway, 1-phosphatidylinositol 3-kinase and NAD(P)H oxidase. As AGE–RAGE signalling is probably cell-type and ligand specific, the array of cellular and tissue responses has to be quite broad. One of the well-described down-stream events following activation of upstream kinases by AGE ligation to RAGE is NF-κB activation. Unlike the physiological transient NF-κB activation promoting cell survival under a variety of stimuli, in diabetes (*in vivo* in mononuclear cells from patients with T1DM as well as *in vitro*) NF-κB activation has been shown to be sustained over long-term periods as a result of overproduction of the NF-κB p65 subunit, while at the same time degradation of the inhibitory subunit of NF-κB is increased[69]. Considerable data relating to the actions of RAGE have been generated using blockade of signalling by soluble RAGE, transfection of cells with a dominant negative RAGE plasmid or transgenic mice expressing RAGE tissue-specifically or RAGE-deficient mice. Interestingly, RAGE also exists *in vivo* in a soluble form similar to other secreted pattern-recognition receptors (such as pentraxins, mannan-binding lectin etc.). There have been several independent descriptions of the existence of endogenous RAGE variants derived by alternative splicing of the gene leading to C- or N-terminal truncation[70–72]. A proportion of the RAGE isoforms produced is tissue-specific, regulated by as yet unknown mechanisms. Circulating C-terminally-truncated soluble RAGE of approximately 50 kDa produced by endothelial cells is regarded as a naturally-occurring competitive inhibitor of signalling pathways induced by the transmembrane RAGE. Following the discovery of soluble RAGE, decreased levels have been demonstrated in several diseases in association with enhanced formation of AGE, such as coronary disease[73], carotid and femoral atherosclerosis[74], essential hypertension[75], diabetic retinopathy[76,77], rheumatoid arthritis[78] and Alzheimer's disease[79].

The 'second' hit: decreased excretion of advanced glycation end products

Processing and metabolism of advanced glycation end products

The integrity of the cellular proteome is maintained by rapid proteolysis of misfolded or altered proteins by the proteasome[80]. Proteasomes are large protein complexes that are located in the nucleus and cytoplasm of all eukaryotes. They can function as the 26S proteasome, which comprises one 20S core particle and two 19S regulatory caps that carry out ubiquitin- and ATP-dependent proteolysis, or as the 20S proteasome alone. Oxidised proteins are degraded directly by the 20S core particle

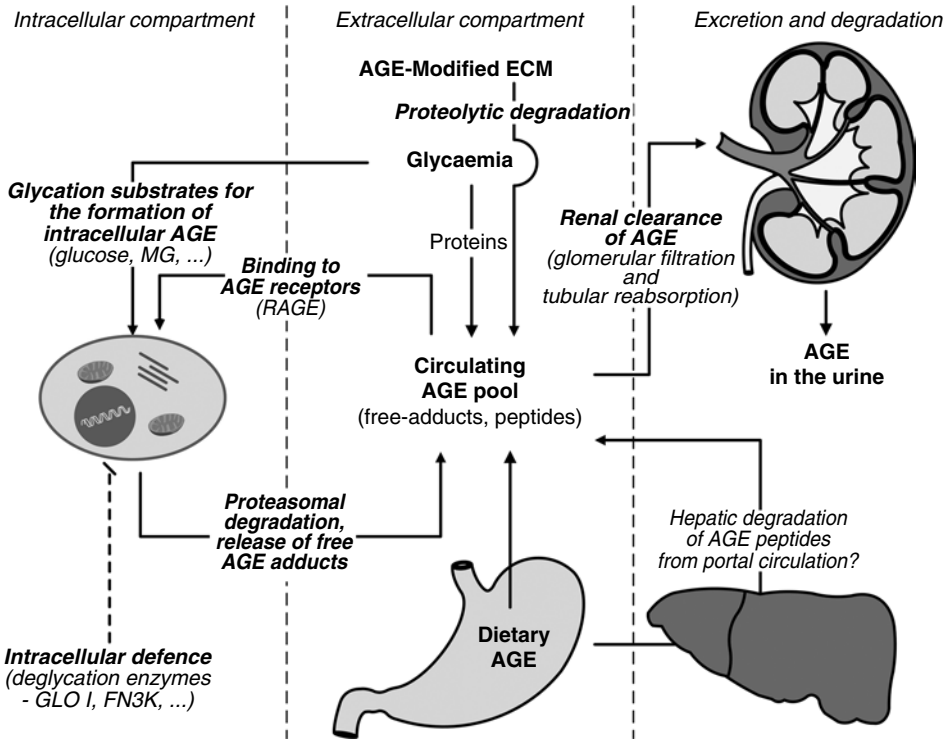

Fig. 1. Overview of advanced glycation end product (AGE) metabolism and factors influencing AGE turnover and the concentration of AGE in particular body compartments. MG, methylglyoxal; GLO I, glyoxalase I; FN3K, fructosamine 3 kinase; RAGE, receptor of AGE; ECM, extracellular matrix.

without the involvement of the 19S regulatory cap and do not require ATP hydrolysis or tagging with ubiquitin[81]. Cellular proteins modified by AGE are also subject to intracellular proteolytic degradation, releasing free AGE into the circulation (i.e. AGE-free adducts)[22], and are probably the most important source of AGE in circulation, even if cellular glycated proteins exhibit some extent of resistance to proteasome degradation[82]. *In vitro* glyoxal treatment of fibroblasts inhibits 20S proteasome peptidase activities without changing the proteasome content[82]. Additionally, a decrease in proteasome degradation can be, to some extent, a result of AGE modification of lysine residues that are then inaccessible for ubiquitinylation or glycation by the proteasome itself[83,84].

In the circulation the total pool of AGE consists of those bound to polypeptides (i.e. formed primarily on plasma proteins), peptides (probably released by extracellular proteolysis from tissue-immobilised AGE) and amino acids (formed by proteolytic degradation of intracellular proteins, direct modification of circulating amino acids and by exogenously-derived AGE, primarily those contained in food and absorbed from the intestine; see Fig. 1). The nomenclature of AGE in the context of their metabolism therefore distinguishes between (1) glycation adduct residues of proteins, (2) glycation adduct residues of peptides (0·5–12 kDa) and (3) glycation-free adducts (amino acids). The latter category represents the most abundant form of AGE in the plasma of healthy subjects as well as subjects with diabetes[35].

Role of the kidney in the excretion of advanced glycation end products

Renal clearance is the predominant means of excretion of AGE, particularly the low-molecular-weight fraction (glycation-free adducts)[35]. The predominant plasma AGE (hydroimidazolones, N_ε-carboxymethyl-lysine (CML) and N_ε-carboxyethyl-lysine) have a high renal clearance[22] and their levels are inversely correlated with renal function[85]. AGE adducts and peptides are filtered into the glomeruli and a small proportion may be reabsorbed and degraded by proximal tubular cells[86]. Subjects with chronic renal failure have increased plasma levels of glycation-free adducts (fructoselysine, methylglyoxal-H, CML and N_ε-carboxyethyl-lysine) compared with normal controls ($\leq$5-fold); however, in patients with end-stage renal disease and renal replacement therapy plasma glycation free adducts are increased $\leq$18-fold on peritoneal dialysis and $\leq$40-fold on haemodialysis[87]. AGE accumulate in patients with uraemia to a much greater extent than in subjects with normal renal function[88–91]; however, AGE do not differ between patients with uraemia associated with diabetes and those with uraemia not associated with diabetes[92,93]. Renal replacement therapy itself also plays a role; the concentration of AGE in the peritoneal dialysate exceeds that of the plasma, suggesting that AGE might be formed *de novo* in the peritoneal cavity from the glucose and its derivatives formed during heat sterilisation of the dialysate[27]. The origin of increased AGE in uraemia is

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complex and not entirely dependent on their decreased renal clearance; it is more likely to be a consequence of enhanced oxidative and carbonyl stress and inflammation induced by uraemic toxins and contact with dialysis membranes and fluids[94,95].

The involvement of the liver in the removal of AGE has also been studied, as preliminary experimental data for animals injected with heavily-modified albumin indicate hepatic uptake by scavenger receptors and metabolism of AGE[96,97]. Similarly, elevation of AGE in subjects with liver cirrhosis and subsequent normalisation after liver transplantation has been demonstrated[98]. However, extraction of AGE formed *in vivo* (minimally modified compared with the previous experiments) from the blood entering and leaving the liver of both healthy subjects and patients with liver cirrhosis does not suggest marked hepatic degradation[99].

Aetiology of chronic renal failure and end-stage renal disease, diabetic nephropathy

Diabetic nephropathy is a serious long-term consequence of diabetes that affects approximately 30% of patients with T1DM and T2DM, and it is the major single cause of chronic kidney disease and end-stage renal disease in developed countries (approximately 28%). Other diseases leading to chronic kidney disease and end-stage renal disease include hypertension (approximately 24%), glomerulonephritis (21%) and several less-common diseases such as polycystic kidney disease, chronic pyelonephritis, nephrolithiasis and systemic lupus erythematosus.

The aetiology of diabetic nephropathy comprises both the metabolic and haemodynamic changes that accompany diabetes[100]. The principal metabolic changes associated with hyperglycaemia have been described earlier (pp. 61–62). Haemodynamic changes are represented initially by increased intraglomerular pressure, which is independent of systemic blood pressure and can be detected quite early after the onset of the disease. Blockade of the rennin–angiotensin system (by angiotensin-converting enzyme inhibitors and/or angiotensin II receptor blockers) has been convincingly shown to delay the onset and progression of diabetic nephropathy, not only by its effect on filtration pressure but also by its non-haemodynamic effects (suppression of ROS production and cytokine release)[100–103]. All parts of nephron (the vasculature, glomerular filtration barrier, tubuli and also the kidney interticium) undergo pathological changes during the course of diabetic nephropathy. Experimental data (mainly from animal studies using inhibitors of advanced glycation) suggest that the pathogenic mechanism in the diabetic kidney is mainly mediated by AGE[104]. Direct modification of renal proteins by AGE may produce changes in charge selectivity, solubility, conformation (e.g. type IV collagen of glomerular basement membrane and its interaction with podocytes via integrins) and cell turnover (mesangium). Moreover, AGE also interact with specific receptors and binding proteins to influence the renal expression of growth factors and cytokines implicated in the progression of diabetic renal disease. Immunohistochemical data obtained from human or rodent kidneys show that RAGE is expressed by podocytes (but not in mesangial cells or glomerular endothelium)[105] and by proximal tubular cells[106]. In rodent models activation of RAGE contributes to glomerular pathology (enhanced permeability, inflammation and glomerular basement membrane thickening), mesangial expansion and tubular changes[106–110]. Engagement of RAGE by AGE (and probably also S100/calgranulins) in the diabetic kidney contributes through the cascade of signalling events using ROS as secondary messengers in the activation of transforming growth factor β, connective tissue growth factor and vascular endothelial growth factor axes directly responsible for renal remodelling. Experimental blockade of AGE formation by benfotiamine[111], pyridoxamine[112] and alagebrium[113,114], blockade of RAGE by neutralising antibodies[115] or the absence of the latter in RAGE-knock-out mice[107] completely suppress structural and functional changes associated with diabetic nephropathy, thereby supporting the pathogenic role of AGE–RAGE interaction in diabetic nephropathy.

The third hit: increased intake of exogenous advanced glycation end products in the diet

Maillard reaction; dietary advanced glycation end products sources

In 1912 the French scientist Louis Camille Maillard described the 'browning' or Maillard reaction between reducing sugars and amino acids during cooking[116]. Products formed by this reaction (Maillard reaction products) contribute to the qualitative properties of foods such as colour, taste and aroma; moreover, it has become increasingly evident that they can also affect the nutritional and toxicological properties of food. The potential biological effect of endogenously-formed AGE, especially in situations like diabetes or uraemia, has also stimulated intensive interest in the potential contribution of dietary AGE to an individual's total AGE levels. Thermal processing of foods enhances their digestibility, sensory properties and shelf life, producing microbiologically-safe products with the desired nutritional quality that make a major contribution to the human diet. Although such treatment can lead to the formation of components with presumably health-promoting properties (such as antioxidants or melanoidins), it can also lead to the formation of potentially-harmful compounds (such as heterocyclic amines, acrylamide and Maillard reaction products)[117]. It is clear that food is a rich source of pre-AGE (mainly fructoselysine) and AGE (mainly methylglyoxal-H, CML, pyrraline, pentosidine). Most dietary AGE are derived from sugar- and protein-rich cooked foods such as bakery products, roasted meat, milk and some other drinks (coke drinks, for example, have a lower AGE content than untreated milk, and the AGE content of milk further increases during pasteurisation and sterilisation[118]), although, the levels depend also on the preparation conditions, presence of metals and water content[119]. Qualitative and quantitative assessment of AGE in food is currently an important area of food research[120].

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Bioavailability, metabolic fate and excretion of dietary advanced glycation end products

It seems that highly-glycated proteins (containing fructoselysines and AGE) may not be digested efficiently (resistance to proteolysis) and that the majority of AGE present in foods are not absorbed from the gastrointestinal tract but excreted in the faeces. More precisely, approximately 10–30% of the ingested AGE are intestinally absorbed, mainly in the form of glycation-free adducts and peptides, and are subsequently found in the circulation[121–123], where the peptides undergo rapid degradation to free adducts, as shown by comparing the concentration of glycated peptides in the portal and systemic venous circulation[99]. Absorbed AGE are excreted rapidly in the urine by subjects or experimental animals with normal renal function, as indicated by balance studies quantifying recovery in the urine of CML, pyrraline and pentosidine from different foods or after injection of labelled compounds[123–126]. In these studies ≤80% of AGE were recovered in the urine, predominantly in the glycation-free adduct form, although it may have been as low as 2% in the case of protein-bound AGE (e.g. pentosidine in the free adducts in brewed coffee *v.* that of pretzel sticks). In animal studies faecal excretion was reported to be ≤26–29% for CML but only 1–3% for fructoselysine[122,123], which was considered to be an effect of the metabolisation of fructoselysine by colon microflora, e.g. *Escherichia coli* can degrade fructoselysine to glucose 6-phosphate and utilise it as a substrate[127]. It is not clear whether AGE are similarly degraded by colon microflora[128].

It is difficult to interpret the findings of both animal and human studies of the bioactivity of AGE because of a series of confounding factors, experimental (study design, duration of administration, medical condition of subjects studied, species studied etc.) as well as methodological (different composition and preparation of high-AGE diet, analytical methods employed to quantify AGE etc.). While some studies have shown a significant effect of dietary AGE, other studies have not found such an effect (see following discussion). Nevertheless, such disparity certainly stimulates further carefully-designed experiments.

Bioactivity of dietary advanced glycation end products: animal studies

The biological effects of dietary AGE have been investigated in healthy rodents fed isoenergetic chow with different AGE contents (for study periods ranging from 6 weeks to 6 months). Rats on a high-AGE diet have been reported to show a higher increase in plasma AGE, urinary excretion of AGE, weight gain, renal protein excretion and renal expression of the pro-fibrotic cytokine transforming growth factor β[129], while, increased fasting plasma insulin has been detected in mice[130].

Similar findings have been obtained in studies of mice with diabetes (non-obese or db/db) fed a high-AGE diet (higher plasma AGE, weight gain and fasting plasma insulin)[131], as well as in another study in which there was also progressive development of diabetic nephropathy (higher urinary AGE excretion, renal protein excretion, glomerular hypertrophy, mesangial expansion and expression of transforming growth factor β) and shorter survival (non-obese NOD mice)[132]. A low-AGE diet has also been found to delay the onset of diabetes in diabetic (NOD) mice[133], improve wound healing in db/db mice[134] and delay the progression of atherosclerosis[135]. In the latter study genetically-hypercholesterolaemic (apoE-deficient) mice fed a low-AGE diet for 4 weeks following experimental injury of the femoral artery were reported to develop a lesser extent of neointimal formation (scar tissue) than animals fed a high-AGE diet[135]. Moreover, in the same animal model rendered diabetic by treatment with streptozotocin, 2-month dietary AGE restriction was associated with lower serum AGE, reduced formation of atheromatous lesions, lower tissue expression of AGE receptors and infiltration with inflammatory cells without concomitant differences in plasma glucose, TAG or cholesterol[136].

The effect of dietary AGE on renal function has been studied using an experimental model of renal failure (subtotally-nephrectomised rats). Nephrectomised rats were found to exhibit higher weight gain, proteinuria, kidney hypertrophy and expression of transforming growth factor β than healthy rats[129]. Administration of alagebrium (AGE breaker) was shown to delay and/or reverse established diabetic nephropathy in db/db mice by reducing systemic AGE and facilitating their urinary excretion[137]. Of particular interest is a study designed to elucidate the potential effect of AGE as a secondary renal insult in rats with a developmental nephron deficit. A low-protein diet fed to female rats during pregnancy and lactation was found to reduce nephron number in their offspring compared with that of the offspring of female rats fed a normal-protein diet; however, no obvious differences were found in systemic blood pressure or glomerular filtration rate. Administration of AGE for 4 weeks beginning at the 20th week of age was shown to induce greater expression of the genes encoding transforming growth factor β and procollagen III as well as renal accumulation of AGE when compared with rats with a normal nephron status[138].

Bioactivity of dietary advanced glycation end products: human studies

In a study of ninety healthy subjects AGE intake (assessed by 3 d food records) was shown to be correlated with circulating AGE, AGE-modified LDL and high-sensitivity C-reactive protein (CRP) concentrations[139]. In a larger study of 172 healthy subjects of different ages it was found that AGE are directly influenced by dietary intake (independent of age or energy intake), and circulating AGE (CML and methylglyoxal derivatives) are elevated in older subjects and correlated with indicators of inflammation and oxidative stress across all ages[140]. A 6-week nutritional intervention study of subjects with diabetes has demonstrated that circulating AGE can be modulated by altering dietary AGE intake, and high AGE intake is associated with higher levels of inflammatory molecules (CRP, TNFα) and markers of endothelial dysfunction

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(vascular-cell adhesion molecule 1)[141]. Furthermore, a comparison of the effects of low and high intakes of dietary AGE on the modification of plasma LDL in patients with diabetes who had equal glycaemic control and lipidaemia has shown that high AGE intake can transform circulating macromolecules to a much greater extent and render them more pro-atherogenic than those of subjects on a low AGE intake[142]. Also, a high-AGE diet was found to induce more pronounced micro- and macrovascular dysfunction and endothelial dysfunction compared with a low-AGE diet in a cross-over study with twenty subjects with T2DM[143]. The same effect (impaired flow-mediated macrovascular dilatation and endothelial dysfunction) was observed in a study in which forty-four subjects with T2DM and ten subjects without diabetes were administered a single oral AGE challenge[144].

When subjects with chronic renal insufficiency were assigned to either a high- or low-AGE diet the low intake was found to be associated with a decrease in AGE, CRP, plasminogen-activator inhibitor-1, vascular-cell adhesion molecule 1 and TNFα levels[145]. The importance of this finding is not clear, however, as a large cross-sectional study of >300 patients on haemodialysis that analysed the relationships between AGE and CRP and all-cause as well as cardiovascular mortality has shown better survival of subjects with high total serum fluorescent AGE and CML levels despite higher levels of inflammation markers (CRP)[146]. The authors suggest that high serum AGE in patients on haemodialysis with better survival is either an epiphenomenon or reflects a better nutritional status. An inverse relationship between circulating AGE and glomerular filtration rate has also been reported for subjects with T1DM as well for subjects without diabetes[85,147]; however, a correlation with inflammatory markers was only present in subjects with diabetes. An even larger study that followed 450 subjects with T2DM and diabetic nephropathy for 2·6 years has ruled out serum CML level as an independent risk factor for cardiovascular or renal outcomes[148].

Interesting findings have been reported from a study of plasma AGE in vegetarians compared with subjects on a typical Western diet. Vegetarians were found to have unexpectedly higher plasma AGE, although not in association with typical AGE toxicity such as induction of insulin resistance (glycaemia), nephrotoxicity (glomerular filtration rate) or inflammation (CRP)[149]. On further investigation of the components of the metabolic syndrome in vegetarians and omnivores[150], the authors have proposed that this finding might be the result of a higher intake of fructose (in fruit), which is a better glycating agent that glucose, and that the potential AGE toxicity may be counterbalanced by higher levels of antioxidants often detected in vegetarians.

Advanced glycation end products *v*. (nutri)genomics and (nutri)genetics

One of the greatest changes preceding and accompanying the current increase in complex diseases has been the change in the human diet, its quality as well as its quantity. Beyond the role of diet as an energy source, micronutrients (vitamins, Ca, Fe etc.) and macronutrients (NEFA, cholesterol, glucose) are potent environmental signals that influence cellular metabolic programming, homeostasis and gene expression. Nutrigenomics is a relatively new discipline that seeks to provide an understanding of how nutrients affect gene expression[151], and studies of the effect of dietary AGE are no exception. Experiments performed *in vitro* with food-derived AGE have confirmed their potent effect, and the previously observed biological effects of AGE could theoretically also apply to food-derived AGE. AGE derived from common thermally-processed foods (animal products, vegetables, starches) have been studied *in vitro* before ingestion and have been shown to possess pro-oxidative (depletion of reduced glutathione, cross-link formation), pro-inflammatory (induction of TNFα) and signalling properties in endothelial cells (human umbilical vein endothelial cells)[152]. Another study has investigated the effect of food compounds formed by heat treatment during processing of food on the expression of the RAGE and p44/42 mitogen-activated protein kinase activation[153]. Dose-dependent activation of RAGE signal transduction pathways was found in response to food-derived AGE and other thermally-produced compounds, as well as inhibition of this activation by pre-incubation with anti-RAGE antibody or in cells expressing C-terminally-truncated RAGE. Nevertheless, because of the limited bioavailability of AGE the true effect of food-derived AGE is probably minor in healthy subjects, although definitely worthy of further investigation in vulnerable groups such as infants and patients with diabetes, uraemia and bowel disease.

Another important issue is to understand how genetic variability in relevant genes modulates the effect of dietary components on specific phenotypes, which is the working definition of nutrigenetics. The current human genome has evolved under environmental influences that were, until recently, predominantly harsh rather than hospitable. The resulting metabolic 'thriftiness' is therefore a logical outcome, but undesirable against a background of affluence. Some of the best examples of the nutrigenetic consequences come from lipid metabolism, i.e. how genetic polymorphisms in genes encoding proteins involved in lipoprotein metabolism modulate the effect of dietary lipid intake on plasma lipid levels or the response to lipid-lowering interventions. In the context of both endogenously-formed AGE and food-derived AGE genes with potential (nutri)genetic importance are (1) those encoding enzymes detoxifying AGE and their precursors (e.g. glyoxalase system or FN3K) and (2) those encoding AGE-binding scavenger and signalling receptors (e.g. RAGE, galectin-3 etc.). The former group, i.e. genetic variability in deglycating enzymes resulting in their functional variability, could contribute to the observed inter-individual variation in AGE levels that was found in a study of twins without diabetes[154]. Approximately 74% heritability of AGE levels (assessed by serum CML) was found, which was independent of heritability of fasting glucose or HbA_{1c}. The notion that genetic factors play an important role in determining AGE levels in the healthy state further emphasises their pathophysiological potential in diseases.

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However, in a study of the common polymorphism A111E in the glyoxalase I in diabetic nephropathy no significant association has been found[155]. The activity of another important deglycating enzyme, FN3K, has been shown to exhibit a wide inter-individual variability influenced by the two polymorphisms in the FN3K gene[156]. Neither enzyme activity nor frequency of the two gene variants was found to differ between groups of subjects with T1DM and without diabetes, although the relationship with diabetes complications was not studied.

The author's group has been involved in the study of genetic variability in the RAGE gene since soon after its discovery, and together with others has contributed to the identification of several common polymorphisms in the RAGE[157] and some of their potentially interesting phenotypic outcomes, e.g. circulating levels of selected non-enzymic antioxidants[158]. Of special interest is the modulation by certain genetic variants in RAGE of the genetic risk of the development of diabetic nephropathy in patients with diabetes, since diabetic nephropathy increases the already-enhanced AGE formation by impairment of their excretion. Using relatively novel approaches to genetic epidemiology suitable for studying complex phenotypes (e.g. haplotype analysis and multi-locus association studies) it has been shown that risk variants in the RAGE gene increase the susceptibility to diabetic nephropathy and accelerate its onset[155,159]. Other studies have also identified RAGE variants as risk factors for diabetic nephropathy[160,161].

Conclusions: the double-hit or, alternatively, triple-hit hypothesis in diabetes mellitus?

Glycation represents the most common type of post-translation modification of protein residues. Methylglyoxal-derived hydroimidazolones are the most abundant AGE both as protein residues and free adducts. AGE formation is increased as a consequence of hyperglycaemia in diabetes. Cells contain enzymic systems to detoxify precursors of AGE (dicarbonyls and fructosamines) and functional insufficiency of these systems (e.g. as a result of genetic factors) can be of critical importance. AGE-modified proteins are subject to proteasomal degradation, free adducts are released from cells into the plasma and, under normal circumstances, are rapidly excreted in the urine. A decline in renal function, e.g. as a result of diabetic nephropathy, leads to retention of AGE and aggravation of their toxicity, which is mediated by direct modification of macromolecules by AGE and also through their binding to the cell surface receptors (RAGE and others). The resulting increase in the expression of pro-inflammatory genes contributes to the development of diabetes complications including diabetic nephropathy. A vicious cycle may then ensue whereby AGE contribute to the development of renal impairment, and once developed renal impairment may then contribute to further accumulation of AGE. Although there have been few studies of the biological effects of dietary AGE, current knowledge indicates that the impact of dietary AGE is relatively low in metabolically-healthy subjects with preserved renal function, because limited bioavailability means that AGE intake represents only a minor contribution compared with endogenously-produced AGE. However, in selected groups such as patients with diabetes (especially those with diabetic nephropathy) and uraemia AGE-rich diet can further aggravate AGE-mediated pathology.

In conclusion, accumulation of AGE is intimately associated with the chronic course of diabetes and represents an example of a self-amplifying pathophysiological mechanism. The data presented support the proposal that AGE act as a multiple-hit agent; although further studies are needed to assess their impact more precisely.

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