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CHAPTER 3

The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceutics to reduce AGE accumulation

A.J. Smit, H.L. Lutgers

Department of Medicine, University Medical Center Groningen, Groningen, The Netherlands

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ABSTRACT
Advanced glycation endproducts (AGE) are a class of compounds resulting from glycation and oxidation of proteins, lipids or nucleic acids. Glycation is the non-enzymatic addition or insertion of saccharide derivatives to these molecules. This leads to the formation of intermediary Schiff bases and Amadori products and finally to irreversible AGE. This classical view has been modified in recent years with recognition of the importance of oxidative and carbonyl stress in endogenous AGE formation. AGE may also have exogenous sources, in certain foods and tobacco smoke. A whole class of specific and non-specific receptors binding AGE has been characterized. Apart from cross-linking of proteins by AGE, the effects of receptor stimulation contribute to the development of chronic complications of conditions like diabetes mellitus, renal failure, and atherosclerosis. Possible interventions to reduce the effects of AGE accumulation include AGE formation inhibitors or breakers, or receptor blockers, but possibly also dietary interventions.
Some of the problems with current assay or diagnostic techniques, and several unresolved issues on the role of AGE in disease will be discussed. Our review will focus on the clinical and pharmaceutical implications of these developments.

INTRODUCTION
Advanced glycation endproducts (AGE) are a diverse class of compounds resulting from a glycation process under the strong influence of oxidative or carbonyl stress. Although AGE were initially considered as a noxious byproduct with an adverse effect on the function of proteins due to increased cross-linking, it has become evident that AGE also bind and interact with several receptors, such as the receptor for advanced glycation endproducts (RAGE) and also scavenger receptors like galectin-3 and CD36. Binding of AGE to such receptors mediates intracellular signalling and induces changes in cellular function, resulting in release of cytokines and growth factors. The AGE-receptor interactions are now considered pivotal in the AGE-induced pathogenetic changes.
AGE accumulate with age on long-lived proteins, but at a more rapid rate in conditions like diabetes mellitus and renal failure. Diabetes and renal failure may serve as examples of increased AGE formation and breakdown, respectively, but in both conditions the additional role of increased oxidative stress is evident. This review will address in more detail the relation between AGE accumulation and chronic complications of these and related conditions. We also review the developments of the different strategies in reducing AGE accumulation. AGE formation inhibitors (aminoguanidine, pyridoxamine and others), AGE breakers (ALT-711) and other approaches to reduce AGE accumulation will be discussed.

SOURCES OF AGE FORMATION IN THE BODY

*Endogenous AGE Formation*

It was long believed that glucose, the type of sugar most abundantly contained in an organism, was biologically inert. However, proteins, lipids and nucleic acids can be modified non-enzymatically by glucose and other reducing sugars [1]. In the early stages of AGE formation, the so-called Maillard reaction, the aldehyde groups of reducing sugars react with amino groups of N-terminal amino acids or epsilon lysine to form Schiff bases, which are then converted by the Amadori process into a more stable ketoamine (Amadori product). In most proteins the source of the amino group is the epsilonamino group of the amino acid lysine. Reducing sugars in solution exist in both open-chain and ring (pyranose or furanose) forms. Only the open-chain structures react with proteins. The proportion of the open-chain forms thus determines the reactivity of the sugars in the Maillard reaction with a rank order of glucose < fructose < ribose, with phosphorylated sugars such as intracellular glucose-6-phosphate, being more potent than their unphosphorylated counterparts [2,3] as well. The reactivity of intracellular, phosphorylated sugars is illustrated by a 13.8 fold increase in intracellular AGE content after 1 week of incubation of endothelial cells in the presence of high glucose [4]. Because the glycation process is concentration-dependent in the early rather than later stages of the Maillard reaction, it is enhanced in hyperglycaemia [5,6]. During AGE formation, reactive intermediate products are formed [7,8]. These reactive
intermediate products are also termed in the literature as: dicarbonyls, oxoaldehydes, reactive carbonyl compound (RCO) or, carbonyl stress, [9]. Well-known examples are methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) [10]. MGO can be formed from oxidative decomposition of fatty acids, but non-oxidative mechanisms (anaerobic glycolysis) predominate in its formation. Mitochondrial overproduction of reactive oxygen species, with consequent inhibition of the glycolytic enzyme GAPDH and increased triose phosphate levels have a central role in the formation of intracellular MGO-derived AGE [11,12]. Overproduction of superoxide by the electron transport chain in mitochondria seems not only to be responsible for increased intracellular AGE formation, but also acts as a common pathogenetic pathway for other mechanisms of hyperglycaemia-induced damage like PKC activation, and polyol and hexosamine pathway induction.

The enzyme glycoxalase may have an impairing role in AGE formation by promoting the detoxification of dicarbonyls, as was recently described by Miyata [13]. AGE formation can also be influenced by reducing compounds such as ascorbate. In vitro studies show that anti-oxidants reduce CML (Nε-carboxymethyllysine) formation. Oxidation of polyunsaturated fatty acids in the presence of proteins can lead to CML formation suggesting that lipid oxidation has a role in AGE formation [14]. In inflammatory conditions like rheumatoid arthritis, increased AGE formation arises without hyperglycaemia. An in vitro study showed that myeloperoxidase produced by activated phagocytes, is able to form reactive carbonyls and CML [15]. Thus, besides reducing sugars and peroxidation of lipids, myeloperoxidase seems to play an additional role in the formation of AGE precursors and AGE. This could contribute to the insights of the pathogenesis of various inflammatory diseases.

As a final point in this paragraph of endogenous AGE formation, genetic factors might also contribute to the course of glycation. In a classical twin study, it was found that the interclass correlations in the clinically well-known Amadori product glycated haemoglobin (HbA1c) were considerably higher in monozygotic twins compared with dizygotic twins, independent of an also demonstrable genetic effect on fasting glucose levels [16]. Another mono- and dizygotic non-diabetic twin study
found that, after adjusting for age, current glucose homeostasis and smoking, 28% of the interindividual variation in lens protein autofluorescence was attributable to hereditary factors (and shared environment for 58%, and non-shared environment for 14% [17]. Thus, there are indications that glycation levels are partly genetically determined, but this area has not been extensively studied so far.

**Exogenous Sources of AGE: Food and Smoking**

The Maillard reaction leading to AGE formation was originally described for heating food. AGE ingested with food are absorbed to a small extent, although the diversity of AGE and assay problems quantification of AGE absorption from food is difficult. Oral bioavailability is estimated to be in the order of 10%, the AGE-induced cross-link formation is resistant to enzymatic or chemical hydrolysis [18]. The accumulation of food derived AGE and alpha-oxoaldehyde AGE precursors has been assumed to contribute to AGE derived deleterious effects. When AGE-modified albumin was administered to healthy nondiabetic rats and rabbits [19], after 2-4 weeks animals displayed diabetes-like vascular complications: a significant increase in vascular permeability, significant mononuclear cell migration in subendothelial and periarteriolar spaces and a defective endothelium-dependent and -independent vasodilatation. Furthermore, glomerulosclerosis and albuminuria, comparable to diabetic nephropathy are seen in control rats receiving daily injection with AGE [20]. Both animal studies showed the deleterious effect of exogenous AGE. An additional human study showed that the daily influx of dietary AGE includes glycotoxins that may constitute an added chronic risk for renal-vascular injury in DM [18]. Dietary restriction of AGE food is discussed below under the Interventions paragraph.

Apart from the direct intake of AGE with food, meals can also influence AGE accumulation and production by excursions in glucose levels. After a meal, diabetic patients can exhibit a hyperglycaemic state, a so-called post prandial glucose excursion (PPGE). These PPGE can even occur when blood glucose is tightly regulated. A study showed that the so-called PPGE correlated well with MGO and 3-DG, two highly reactive precursors of AGE formation [21].
Tobacco smoke contains products that produced AGE-like fluorescence, protein crosslinks, mutagenicity and increased AGE on plasma proteins [22]. Cigarette smoke contains high concentrations of GO and MGO from the thermal decomposition of saccharides. These are the likely important mediators of smoking associated AGE formation. Increased serum AGE levels were found in diabetic smokers compared to diabetic non-smokers [23].

REMOVAL OF AGE

The removal of proteins cross-linked with AGE depends on both the degradation of AGE linked proteins to AGE-peptides by macrophages, and on the clearance of AGE and AGE-peptides by the kidney [24-27]. Macrophages with special AGE receptors engulf and subsequently transport AGE. Miyata et al. provides evidence for filtration of the AGE-compound pentosidine through the glomeruli and the active reabsorption of pentosidine in the proximal tubules [26]. After modification or degradation in the proximal tubules pentosidine was finally cleared in the urine. These findings were confirmed for AGE-peptides by Gugliucci et al. [27]. A fall in renal glomerular or tubular function thus affects the capacity for AGE removal and result in higher blood level of AGE and eventually tissue AGE accumulation.

Scavenger receptors in the liver Kupffer and endothelial cells may also result in endocytic uptake of AGE proteins from the blood [28,29]. High serum AGE levels have been reported in patients with liver failure [30]. The expression of the macrophage scavenger receptor (MSR) which is involved in the endocytic AGE uptake in liver endothelial cells, but perhaps also subsequent steps like lysosomal degradation, may be increased by insulin [29]. It was recently reported that plasma AGE levels are markedly elevated in liver cirrhosis and correlated with the severity of disease, while serum CML levels correlated inversely with residual liver function [30]. Elevated plasma CML levels markedly fell within 3 months after liver transplantation. This suggests that the liver also has a function in removal of plasma AGE [31]. Perhaps this occurs with the OST-48 or galectin-3 receptor, or the scavenger receptor (SR)-A or -B for all of which expression in the liver has been reported [32-34].
As for the rate of AGE removal after restoring normoglycaemia (by islet transplantation) in previously diabetic animals, the results of Sensi et al. suggest that reversal of accumulated tissue AGE takes many months to occur [35]. This may explain discrepancies between circulating and tissue AGE levels which have been reported, for example after kidney transplantation [36].

**RATE OF AGE ACCUMULATION**

Equilibrium levels of the reversible Schiff base and Amadori products are reached within hours and days, respectively. AGE form over longer period of weeks but remain irreversibly bound to amino groups. Using both immunohistochemical methods and four monoclonal antibodies for specific AGE molecular structures (including CML and carboxyethyllysine (CEL), but also as yet unknown epitopes), Ling et al. identified in various organs in fetal rats already at 10 fetal days with increasing accumulation during ontogeny [37]. The results suggest that CML as non-fluorescent, non-cross-linked AGE starts earlier to accumulate than fluorolink, a fluorescent, cross-linked AGE. Thus AGE accumulation starts very early, already in the fetal period. The Maillard or browning reactions that eventually lead to cross-linking and denaturation of proteins may occur with all proteins in the body, but long-lived proteins such as collagen, which represents over 30 percent of body protein, accumulate chemical damage with age. Other long-lived proteins include lens-proteins and nerve myelin, which together with collagen are the most important targets for AGE formation. Next to these substrates other forms of macromolecules containing a free amino group are susceptible to AGE formation, like lipids and nucleic acids [38]. Especially in uraemia, associated with very high AGE levels, such shorter lived compounds as lipid constituents are affected. It must be pointed out that the dynamics of circulating AGE may be quite different: in rats with acute renal failure CML levels (measured fluorometrically) increased threefold within 48 hours [39]. The various pathways in the formation and removal of AGE are summarized in Figure 1.
EXAMPLES OF AGE

As mentioned before, the information on the chemical structure of AGE is limited. Of the several dozens of AGE moieties only a minority has been characterized, even less have been related to clinical human disease. In 1984 Pongor et al. first proposed a structure for AGE. They suggested the fluorescent aromatic heterocyclic compound 2-(2-furoyl)-4-(5)-(2-furanyl)-1-H-imidazole (FF1) generated through a condensation of two Amadori products [40]. They also emphasized that FFI is likely to constitute less than 1% of total AGE present in vivo. Sell and Monnier [41] identified a fluorophore present on human dura mater collagen. This fluorophore was identified as an imidazo-(4,5-b)-pyridinium ring with lysine and arginine side chains, named pentosidine. Two other pyrrole-related compounds have now been identified. These are 1-alkyl-2-formyl-
Clinical relevance of AGEs

3,4-diglycosylpyrrole (AGFP) and pyrraline (5-hydroxymethyl-1-neopentylpyrrole-2-carbaldehyde). CML, pentosidine and hydroimidazolone are known to be glyoxidation products which means that they require oxidation reactions for their formation from glucose. As these last three compounds are well characterized they are frequently used as marker for AGE levels in plasma and in histological studies. The structure of pentosidine and CML, but also of some other AGE like pyrraline, CEL and imidazolone has been elucidated. A characteristic property of several AGE is their fluorescence. CML is, however, non-fluorescent. CML may be a dominant AGE antigen in tissue proteins [42,43]. The known AGE are immunologically distinct and coexist on different carrier proteins such as collagen, albumin, haemoglobin, lens crystalline, but also LDL cholesterol. Pentosidine and CML are well-known examples of advanced glycoxidation products, resulting from combined glycation and oxidation. In blood 90% of pentosidine and CML are bound to protein with 10% being free. Under normal conditions Hb-AGE accounts for 0.42% of circulating haemoglobin but this increases to 0.75% in diabetic subjects [44]. Hb-AGE concentration responded slowly to intensive glycemic control and may reflect glycemic control over the preceding 10-14 weeks rather than the 6-8 weeks indicated by HbA1c [45]. In early studies, using ELISA for total AGE, up to 100 fold increases were reported in serum AGE in diabetics with end stage renal disease (ESRD), in later studies 23-fold increases in serum pentosidine in ESRD [46].

Nagai et al. recently described a new precursor of CML, glycolaldehyde (GA)-pyridine, which was present in the cytoplasm of foam cells and extracellularly in the central region of atheroma in human atherosclerotic lesions [47].

BIOLOGICAL EFFECTS OF AGE

Changed Protein Function by Conformational Changes, the Role of Cross-Linking

A common consequence of AGE formation is covalent cross-linking, mostly to proteins. These cross-links may result in entrapment of proteins, lipoproteins and immunoglobulins [2]. Cross-linking of collagen proteins, for example, contributes both to the rigidity and the loss of elasticity of tissues, and increases resistance to proteolysis.
The latter process inhibits tissue remodelling. Among the consequences are the thickening of capillary basement membrane observed in diabetes. This protein modification is also responsible for crystalline lenses becoming opaque in cataracts, a degenerative disease that is frequent in diabetic or aged persons. Glomerular sclerosis is another consequence. These processes are discussed in more detail below.

Small soluble AGE peptides released into the circulation by macrophages after degradation of AGE proteins may react and covalently bind to proteins such as collagen and LDL. Such reactive intermediates from degraded AGE can form, second generation, AGE and so actively contribute to the biological AGE effects.

AGE-formation on the extracellular matrix results in a decreased elasticity and increased thickness and rigidity of the vascular wall and in narrowing of the vessel lumen.

Diminished arterial elasticity in humans with diabetes was related to enhanced AGE-formation before [52]. AGE formed on vascular matrix proteins mediate defective endothelium-dependent vasodilatation by quenching nitric oxide [53]. Moreover, incubations of rat aorta segments with high-glycosylated human haemoglobin inhibited endothelium-dependent relaxation in this vessel [54]. At the microvascular level, the toxic effect of AGE on retinal capillary pericytes and endothelial cells was shown in vitro [55]. The functional effects of AGE on the endothelium include inhibition of prostacyclin production and increased plaminogen activator inhibitor 1 (PAI-1) and vascular endothelial growth factor (VEGF) production [56,57]. AGE also induce the expression of the potent vasoconstrictor endothelin-1 [58]. Part of these latter effects may be due to more effects mediated by AGE receptors than by conformational changes, as further discussed below.

Lipoprotein modification by AGE contributes to the dyslipidaemia frequently observed in diabetic patients [1]. Such a modification of LDL in diabetes may render the LDL particles more atherogenic, contributing to the atherogenic risk for diabetic patients. Another consequence may be that lipoproteins are trapped by AGE formed on the matrix components of the vascular wall, resulting in an impaired cholesterol efflux and subsequently in vascular lipoprotein accumulation [59]. This has, however, been
debated by others [60]. Glycated LDL also triggers endothelial transcription activators like STAT-5 [61].

**Induction of Oxidative Stress**

AGE binding to cellular binding sites results in depletion of cellular antioxidant defense mechanisms such as vitamin C and glutathione and the generation of oxygen free radicals [62,63]. These mechanisms may also involve receptor stimulation. For example, activation of NADPH oxidase in AGE-RAGE mediated generation of reactive oxygen species in human endothelial cells [64]. Semicarbazide-sensitive amine oxidase (SSAO) may have a contributory role by production of MGO [65]. Other experiments showed a potentially important role for oxygen free radicals and NO in mediating permeability and blood flow changes induced by AGE involving increased protein kinase C activity and VEGF production [66]. An additional study confirmed increased VEGF expression by AGE-albumin through the activation of hypoxia inducible factor-1 (HIF-1) [67]. In diabetes, persistent hyperglycaemia causes increased production of free radicals via the processes of autoxidation of glucose and non-enzymatic protein glycation, discussed above, and via an enhanced flux of glucose through the polyol pathway [68]. Oxidation of plasma lipids may stimulate autooxidative reactions of sugars, enhancing damage to both lipids and proteins in the circulation and the vascular wall. In this way, the cycle of oxidative stress and damage is continued and reinforced. Bucala et al. first showed the effect of advanced glycosylation on lipid oxidation [69]. Its role in accelerating atherosclerosis has been discussed by Vlassara and by Bierhaus [2,70].

**Receptor Stimulation and Post-Receptor Effects of AGE**

AGE formation not only changes the physiological properties of proteins and other molecules, but also induces cellular dysfunction through interaction with more or less specific cell surface receptors. An increasing number of AGE-receptors has been identified and characterized. These receptors include the receptor for AGE (RAGE),
AGE-R1, AGE-R2 and AGE-R3, but also other receptors classified as scavenger receptors (SR), SR-A and SR-B.

RAGE was identified in 1992 as a 35-kDa protein in bovine lung endothelial cells, and is still considered as a representative AGE receptor on endothelial cells. It belongs to the immunoglobulin superfamily [71,72]. In contrast to scavenger receptors, binding of AGE to RAGE does not result in endocytosis of AGE, but results by intracellular signalling in, abnormally sustained, activation of NF-kappa-B, and subsequent expression of endothelial adhesion molecules and tissue factor [73,74]. Epithelial-myofibroblast transdifferentiation, an important step in the development of tubulointerstitial fibrosis which is often observed in diabetic nephropathy, is caused by AGE-RAGE interactions [75]. Enhanced RAGE expression also has been correlated with nephropathy, retinopathy, neuropathy, autoimmune, and inflammatory disorders. In the vasculature RAGE has been associated with the induction of apoptotic death [76]. This action may be responsible for several complications related to diabetes, including vascular leakiness and nonresponsiveness. Animal studies using soluble RAGE to block the RAGE receptor also show a reduction in vascular permeability and a suppression of vascular lesion formation. RAGE antibodies were also found by Esposito et al. to prevent an increase in adhesion of peripheral blood mononuclear cells during hyperglycaemia [77]. Not only AGE but also amphoterin and amyloid beta-peptide are major ligands for RAGE. Amyloid beta-peptide is thought to be important in the pathogenesis of Alzheimer’s disease; the role of AGE in this disease is discussed below. At least 9 polymorphisms of the RAGE gene exist. One of these polymorphisms (-429 C allele) has an increased prevalence in type 2 diabetes patients with compared to those without retinopathy [78]. A low expression of the AGE-R1 gene in macrophages from diabetic (type 1 like) mice, but also in fresh peripheral blood mononuclear cells and in Epstein-Barr virus (EBV)-transformed cells from type 1 diabetic patients with complications, together with elevated serum AGE levels, suggests that the normal function of AGE R1 receptor in the turnover of AGE may be ineffective in these patients, possibly by genetic influences [79]. Double transgenic mice with both overexpression of human RAGE in vascular cells, and development of insulin-
dependent diabetes had renal histological changes and increased serum creatinine and albuminuria. The development of histological and functional diabetic nephropathy was prevented with the AGE-inhibitor OPB-9195 [80]. Thus, the functional consequences of RAGE binding depend not only on AGE levels, but also on variations in gene polymorphism and expression. Galectin-3 is a lectin-like protein that has been identified as a component of p90, a 90 kDa AGE-binding protein, originally identified in the rat liver [81]. Galectin-3 modulates cell adhesion, cell cycle control, and mRNA splicing. Galectin-3 may have a protective role against diabetic glomerulopathy, as suggested by the increased AGE accumulation and accelerated glomerulosclerosis in galectin-3 knockout mice made diabetic [82].

AGE which are negatively charged have been shown to bind to SR-A type I and II. In these experiments AGE-BSA underwent active endocytosis upon binding to SR-A [24]. Remarkably, CML does not bind to SR-A while glycolaldehyde-modified proteins which generate CML do [47]. CD36, a highly glycosylated protein of 88kDa, binds various ligands such as fatty acids, collagen, oxidized LDL and has recently been shown to act also as an AGE receptor [83]. CD36 acts in adipocytes as a fatty acid transporter and in macrophages as a receptor for oxidized LDL [84]. CD36 is markedly expressed in the core of atherosclerotic lesions. CD36 is considered as an important oxidized LDL receptor. AGE-binding to this receptor may contribute to the acceleration of atherosclerotic lesions. Apart from modification of LDL-cholesterol by LDL-AGE-formation which is much slower degraded in macrophages, as discussed elsewhere in this paper, and apart from the effects on CD36 as an important receptor for oxidized LDL, AGE may also more directly affect cholesterol metabolism by binding to another scavenger receptor, the SR-B1. SR-B1 is very similar, if not identical, to the one that internalizes acetyl LDL (and oxidized LDL) [34]. SR-B1 also recognizes high density lipoprotein (HDL) and mediates its uptake [85]. SR-B1 is considered to accelerate reverse cholesterol transport by promoting cholesterol efflux from peripheral cells, for example from macrophages in the vascular wall, and mediates selective uptake of HDL-cholesterol by hepatocytes. Ohgami et al. showed that SR-B1 recognized AGE as a ligand. No cross-competition as ligands seems to
exist between HDL and AGE-binding proteins. However, intracellular processing of HDL may be affected by an interaction between SR-B1 and AGE [59]. AGE also affect SR-B1 mediated cholesterol efflux but not SR-B1 independent cholesterol efflux. Thus AGE seem to inhibit reverse cholesterol transport [86].

Expression of the different AGE receptors has been found on a wide range of cell-types including monocytes, macrophages, endothelial cells, mesangial cells, fibroblasts, smooth muscle cells and tubule cells [64,74,79,87].

MARKERS OF AGE AND PROBLEMS WITH CURRENT ASSAYS
Because the structure of AGE is complex and heterogeneous, measurement of tissue and serum AGE has been difficult, and, as there is no recognized standard, different groups may not be measuring in exactly the same way. Reproducibility of many assays is rather poor. The limited knowledge on the specific structure of different AGE moieties also makes it difficult to draw any firm conclusions on the relative toxicity of different specific AGE. Table 1 summarizes the different markers of AGE and their advantages and disadvantages. Classically fluorescence is the method used in many clinical studies as a marker of AGE. AGE has a yellow brownish pigmentation with a characteristic fluorescence pattern: excitation in the range 350-390 nm and fluorescence emission at 440-470 nm. Applying fluorescence spectroscopy, AGE have been measured in serum, urine and also in different kinds of tissues [88-90]. The presence of other, non-AGE, fluorescent substances like NADPH and of non-AGE protein adducts, such as glucose- or lipid-derived oxidation products, that exhibit similar fluorescence spectra, as well as the interference by non-protein tissue components, makes the specificity of this method low. Fluorescent quenching associated with biological samples may also impede specificity. Furthermore, many but not all AGE have fluorescent properties. Examples of non-fluorescent AGE are CML and pyrraline [91,92]. The fact that most clinical data on the relation with AGE accumulation have still been based on association with fluorescence makes this method still valuable. However, tissue samples are required, which makes this invasive
Table 1. Markers of AGEs and their methods. The advantages and disadvantages of the different AGE assessment methods are presented in the third and fourth column of the table.

<table>
<thead>
<tr>
<th>Method</th>
<th>Marker</th>
<th>Compartment</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td><strong>Fluorescence</strong></td>
<td>Collagen linked fluorescence (CLF): excitation 370 nm, emission 440 nm</td>
<td>tissue samples (skin, kidney, cartilage)</td>
<td>proven association with (diabetic) complications</td>
<td>- invasive, thus not feasible for frequent AGE monitoring</td>
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<td></td>
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<td></td>
<td>- interference of non-AGE fluorophores, no detection of non-fluorescent AGE</td>
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<td></td>
<td>Autofluorescence: excitation 360-370 and emission 420-600 nm</td>
<td>in vivo skin</td>
<td>non-invasive, rapid method, therefore feasible for repetitive AGE assessments</td>
<td>- clinical relevance not proven yet</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- interference of non-AGE fluorophores, no detection of non-fluorescent AGE</td>
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<tr>
<td><strong>HPLC</strong></td>
<td>Pentosidine, CML, etcetera</td>
<td>plasma</td>
<td>little invasive, practicable for AGE monitoring</td>
<td>- time consuming, expensive</td>
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<tr>
<td></td>
<td></td>
<td>(tissue)</td>
<td></td>
<td>- correlation with tissue AGE-accumulation unclear</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>- only applicable to AGE with known biochemical structures</td>
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<tr>
<td></td>
<td>Hb-AGE</td>
<td>plasma</td>
<td>little invasive, practicable for AGE monitoring</td>
<td>- correlation with tissue AGE-accumulation unclear, because of:</td>
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<td></td>
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<td>- insufficient representative for AGE bound to other proteins</td>
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<td></td>
<td></td>
<td>- relative short half-life of hemoglobin</td>
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<tr>
<td><strong>ELISA</strong></td>
<td>Pentosidine, CML, etcetera</td>
<td>serum, urine (tissue)</td>
<td>little invasive, low-cost, practicable for AGE monitoring</td>
<td>- correlation with tissue AGE-accumulation unclear</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>- low sensitivity</td>
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<td>- low reproducibility between different laboratories</td>
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<tr>
<td><strong>Collagen crosslinking</strong></td>
<td>protein cross-linking index</td>
<td>tissue samples</td>
<td>provides an indirect quantitative measure of tissue AGE accumulation</td>
<td>- low specificity</td>
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<tr>
<td></td>
<td></td>
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<td>- only valid to collagen rich tissue</td>
</tr>
<tr>
<td></td>
<td>differential scanning calorimetry</td>
<td>tissue samples</td>
<td>quick quantitative method for assessment of in vitro collagen crosslinking, also applicable in tissues with less collagen</td>
<td>- insufficient specificity for in vivo application, therefore clinical relevance unlikely</td>
</tr>
</tbody>
</table>
technique less suitable for repetitive AGE monitoring. Recently, Meerwaldt et al. reported on a rapid, non-invasive measurement of skin autofluorescence to assess AGE accumulation [93]. Although the above mentioned limitations of fluorescence still are valid, this non-invasive technique is much more convenient. If a relation with clinical data and its prognostic value are confirmed, this method might offer a feasible method in patient care.

AGE with known structures, such as pentosidine and CML, can be measured with a high degree of specificity using HPLC [94,95]. More sophisticated methods like GCMS and LC-MS became recently available for the determination of AGE. These methods are, however, quite time-consuming, require considerable training and, are thus expensive. Furthermore, the AGE with a known biochemical structure might only account for a small portion of the total amount of circulating and tissue AGE. Whether the AGE with known structure are clinically relevant is not sure.

Less time intensive, cheaper and more feasible for clinical use, are immunoassay methods, using antibodies against AGE. Nakayama first produced and characterized antibodies raised against keyhole limpet haemocyanine-bound AGE [96]. The result was a polyclonal anti-AGE antiserum that was used in an ELISA. Also, an immunochromatographic detection of AGE in vivo was developed using antibodies raised against bovine serum albumin (BSA)-AGE or RNase-AGE [97,98]. In this way, AGE could be detected that were immunochromatically different from characterized AGE such as CML, pentosidine and pyrraline. Thus far it remains uncertain whether these antibodies can identify all AGE produced in vivo. A common structure in AGE modified macromolecules that interacts with polyclonal antibodies has been suggested [97]. But, polyclonal antibodies against AGE-BSA or AGERNase failed to recognize CML which is claimed to be a dominant AGE in human serum albumin and in tissue proteins [99]. Another problem may be that factors in human serum interfere with the measurement [100]. Roche Diagnostics (Penzberg, Germany) has developed a commercially available competitive ELISA, using an anti-CML monoclonal antibody 4G9 [101,102].
Although many different antibodies have been produced, reproducibility and sensitivity remains a substantial problem. This is probably due to a lack of a well-defined set of antigens. Before antibody assays can be used as a standard, reproducible synthesis of well-defined antigens should be attained. A recent report demonstrated that shortterm heating, as used in the heat-induced epitope retrieval technique in formalin-fixed, paraffin-embedded tissue sections, results in artificial AGE formation and profoundly affects CML formation. This may also have served as an artefact in immunohistochemistry studies [103].

A competitive radioreceptor assay based on AGE-specific receptors on the macrophage-like tumour cell line RAW 264.7 was developed earlier, but is also not widely used, possibly due to the strong interference in this assay with polyanions such as heparin [104].

As an indirect measure of AGE, collagen crosslinking has been proposed. The extracellular matrix protein collagen is highly prone to AGE-formation. It has been shown that the susceptibility of collagen to digestion by pepsin provide an index of protein cross-linking [91,105]. In this method, often tissues are used with high amounts of collagen, like rat tail tendon, to increase specificity of the measurement. Thus, this method only provides an indication of tissue AGE accumulation. Moreover, some AGE such as CML and pyrraline do not occur as crosslinks in proteins. Applying differential scanning calorimetry (DSC), crosslinking can also be determined in tissues with less collagen. Although this method will provide a quantitative method for determining the crosslinking of collagen in vitro, it lacks the specificity to assess crosslinking in vivo. Mentink et al. showed that DSC was able to detect a lower rate of crosslinking by aminoguanidine in rat tendons (but not in rat skin) [106]. However, no effect of the AGE breaker ALT-711 was found in either tissue with DSC. The advantage of DSC is that it is quick and can be well quantified in comparison with methods like enzyme digestibility.

Miyazaki recently attracted attention to the problems associated with preparing AGE-BSA (bovine serum albumin) complexes that are used in ligand experiments for scavenger receptors. Their preparations belong to the extensively modified preparations
in comparison to those from some other laboratories [86]. Marked differences in ligand activity to scavenger receptors may exist even when specific AGE like CML are present in both of them. They suggested that the presence of glycolaldehyde may be important for binding behaviour of AGE preparations to receptors.

Subramaniam warned against the widely used dichlorofluorescein assay to assess cellular oxidant stress by microtiter plate assay because of MGO effects [107]. Hui et al. found that trace amount of redox-active metal ions in biological buffers may induce oxidative stress and alterations in cellular functions which would otherwise be possibly ascribed to AGE-proteins [108].

AGE IN DISEASE
A dynamic equilibrium seems to exist between AGE circulating in blood, AGE accumulating in tissue and renal (and perhaps hepatic) clearance of AGE. It is no surprise that AGE accumulation exceeds normal in patients with diabetes mellitus or renal failure [109-112]. The association with various chronic complications of these diseases is discussed below in more detail. AGE have also been reported to accumulate in amyloid associated diseases such as dialysis related amyloidosis and Alzheimer Disease, and in the plaques found in atherosclerosis. In the neurological field increased local AGE accumulation has also been reported in amyotrophic lateral sclerosis. Accumulation of AGE in cartilage has been linked to synovial inflammatory diseases, including rheumatoid arthritis and osteoarthritis. Table 2 shows an overview of all diseases discussed in this paragraph with related references.

**AGE Accumulation in Diabetes Mellitus (DM) and Chronic Complications**
Increased concentrations of AGE have been associated with chronic complications in cross-sectional studies in DM: AGE (CML, pentosidine, others) content of skin collagen has been shown to be a risk marker for microvascular complications of diabetes, independent of actual glucose or recent HbA1c values [113]. Serum AGE are increased in diabetic patients compared to controls [46,114,115]. AGE concentrations in serum, but also in kidneys, skin and vascular tissue may rise within 5 weeks after
Clinical relevance of AGEs

Table 2. Overview of diseases or complications in which AGE accumulation has been described in the literature to play a pathogenetic role.

<table>
<thead>
<tr>
<th>Diseases with increased AGE accumulation</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Diabetes Mellitus</strong></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>35, 47, 116, 120, 167</td>
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<tr>
<td>Retinopathy, cataract</td>
<td>6, 121-132</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>46, 80, 90, 121, 133-136</td>
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<tr>
<td>Neuropathy</td>
<td>137-142</td>
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<tr>
<td>Periodontitis</td>
<td>191, 192</td>
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<tr>
<td><strong>Renal failure</strong></td>
<td></td>
</tr>
<tr>
<td>Peritoneal dialysis</td>
<td>3, 147, 151</td>
</tr>
<tr>
<td>Hemodialysis</td>
<td>3, 150, 152-156</td>
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<tr>
<td>Kidney transplantation</td>
<td>36, 163-165</td>
</tr>
<tr>
<td>Dialysis related amyloidosis</td>
<td>76, 157-159</td>
</tr>
<tr>
<td>Disturbed bone metabolism</td>
<td>160, 161</td>
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<tr>
<td><strong>Atherosclerosis</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Alzheimer disease</strong></td>
<td>109, 114, 166-174</td>
</tr>
<tr>
<td><strong>Amyotrophic lateral sclerosis</strong></td>
<td>175-182</td>
</tr>
<tr>
<td><strong>Arthritis (Osteoarthritis, Reumatoid arthritis)</strong></td>
<td>183, 184</td>
</tr>
<tr>
<td><strong>Amyloidosis</strong></td>
<td>185-187</td>
</tr>
<tr>
<td><strong>Amyloidosis</strong></td>
<td>188-190</td>
</tr>
</tbody>
</table>

making an animal diabetic, for example with STZ [96]. Serum AGE levels are higher in both type 1 and type 2 DM, are already increased early in the disease, as shown in prepubertal diabetic children to approximately 1.5-fold, and are associated with the severity of retinopathy and nephropathy, but also with coronary heart disease in type 2 diabetes mellitus patients [116]. Hb-AGE rises from 0.42 to 0.75% in diabetic subjects [44]. In patients with DM combined with ESRD serum AGE levels were increased 100-fold compared to diabetics with normal renal function, in another study serum pentosidine was increased 23-fold in DM with ESRD [46,117]. Comparable rank orders for subjects with DM and ESRD, DM without renal disease and controls have been reported for arterial wall AGE content [46]. Vascular wall AGE content has been semi-quantitatively found to be increased in subjects with DM and vascular disease, being present in fatty streaks and macrophages [118].

When exogenous AGE are administered to rats to attain plasma levels equivalent to those in diabetic rats the renal AGE content was 50% greater than in control rats after 5 months, accompanied by a glomerular sclerosis with a 50%
expansion of the glomerular volume, and with an increase in mesangial matrix and capillary basement membrane thickness [119].

Sensi et al. investigated the effects of restoring normoglycaemia 4 months after homologous islet cell transplantation in different organs of rats previously made diabetic shortly or for longer periods before transplantation [35]. Only after a longer (8 months) preceding period of DM AGE content was elevated in all organs. In the eye lens but not in the aorta AGE content was lower than in nontransplanted diabetic control rat. This suggests that in DM prolonged time periods are an essential prerequisite not only for the accumulation of AGE to develop, but also to be reversed.

In addition to the earlier discussed biological effects, AGE accumulation may also contribute to dyslipidaemia in diabetes both by affecting the HDL receptor and by AGE-modified LDL [1]. The modification of LDL in diabetes may render the LDL particle more atherogenic, thereby increasing the atherogenic risk for diabetic patients. Park and co-workers showed that administration of the soluble extracellular domain of the receptor for AGE (RAGE) completely suppressed diabetic atherosclerosis in STZ-diabetic apolipoprotein E-deficient mice, in a manner independent of the level of glucose and lipids [120].

Diabetic Retinopathy and Cataract
Background diabetic retinopathy has developed after 25 years in almost all patients with DM, a much lower percentage has then developed proliferative retinopathy (8 to 26%). CML and pentosidine skin collagen accumulation is associated with the severity of retinopathy in type 1 diabetes patients, independent of age and diabetes duration [6]. In an autopsy study CML was present around retinal blood vessels in diabetics, but not in controls. Furthermore it is shown that formation of AGE measured by ELISA in skin biopsies are preceded and correlated with early manifestations of retinopathy as well as nephropathy [121]. Immunohistochemical studies in rats have revealed that AGE (CML) reactivity was increased in diabetic retinas [122-124]. No difference was present between subjects with DM with proliferative retinopathy and those with background retinopathy [125]. In two diabetic animal studies the AGE formation
inhibitor aminoguanidine (see below) prevented microaneurysm formation, pericyte loss, and the development of accelerated diabetic retinopathy, respectively [126,127]. Also the development of DM-related abnormalities of the retina could be prevented by aminoguanidine, indicating a prominent role for AGE in this complication [122].

Glycation of lens crystalline, and subsequent oxidation, may play a major part in accelerated cataract formation in DM. Alterations in Na+/K+-ATPase activity due to glycation may contribute to this. Evidence for AGE formation has also been found in the vitreous body using an AGE-specific ELISA, and was found to be related to age and the presence of DM [128]. In the vitreous body of patients with diabetic retinopathy and in a nondiabetic control group the increased levels of pentosidine in diabetics are strongly related to VEGF and other cytokines [129]. AGE accumulation has also been observed in the cornea of diabetic patients [130-132]. The formation of AGE on fibronectin and laminin in Descemet’s membrane in the cornea of the eye inhibited the attachment and spreading of endothelial cells. This may contribute to the increased prevalence of corneal endothelial abnormalities in patients with DM [131]. Thus, several lines of evidence indicate a role for AGE in the development of several forms of diabetes related eye disease, especially retinopathy, with a favourable effect of aminoguanidine.

**Diabetic Nephropathy**

Diabetic nephropathy develops in approximately 40% of patients with DM. Multiple studies have shown that AGE are important factors in the pathogenesis of nephropathy. CML, pyrraline and pentosidine have all been found in increased quantities in kidneys of patients with DM with or without ESRD [133]. AGE accumulation is related to the severity of diabetic nephropathy. Animal studies in rats have immunohistochemically shown AGE accumulation in the glomerular extracellular matrix. Expression of proteoglycans which form an important constituent of the kidney extracellular matrix is decreased in cultured kidney epithelial cells at CML-protein concentrations as found in diabetes [134]. In fact, the hyaline depositions in the classical Kimmelstiel-Wilson lesions of diabetic kidneys consist for a major part of AGE. Exposition to AGE-
crosslinked proteins results in increased oxidative stress in rat mesangial cells and in an increase in protein kinase C activity. Makita et al. showed that the increase in circulating AGE-peptides of diabetic patients correlates with the severity of renal function impairment [46]. Deuther-Conrad highlighted the toxic effect of the AGE-peptides in cultured kidney epithelial and proximal tubular cells [135]. Overexpression of RAGE in transgenic diabetic mice resulted in histological and functional changes of diabetic nephropathy [80].

Pharmacological inhibition of AGE-formation by aminoguanidine prevented the development of kidney lesions, albuminuria and mesangial expansion in diabetic rats [90, 136]. In human studies discussed in more detail below, aminoguanidine 150 mg twice a day reduced proteinuria significantly in a large (690 patients) study in patients with type 1 DM and moderate renal impairment. As mentioned before, Beisswenger and colleagues showed that formation of AGE measured by ELISA in skin biopsies preceded and correlated with early manifestations of retinopathy as well as nephropathy [121].

**Diabetic Neuropathy**

Using sensitive methods the large majority of patients (80-90%) with DM will eventually develop symptoms or signs of diabetic polyneuropathy, a much smaller percentage develops autonomic neuropathy. The important role of glycation in the development of diabetic neuropathy has been recently reviewed [137]. In vitro, diverse mechanisms involving AGE in neuropathy seem to play a pathogenetic role: accumulation of AGE in vasa nervorum results in wall thickening, occlusion and ischemia of nerves, and consequent myelin damage with segmental demyelination. Glycation of the axonal cytoskeletal proteins such as myelin and neurofilament results in slowed axonal transport and axonal degeneration [138]. Nerve fiber regeneration may be reduced because the function of nerve growth factor and other growth factors like fibrin, but also because laminin in Schwann cell basal laminas is glycated and impeded in its function [139]. In animal studies aminoguanidine prevented AGE-induced reductions in peripheral sensory nerve conduction velocity and action
Clinical relevance of AGEs

potentials [140,141]. In diabetic humans more CML is found in the perineurium, in pericytes of endoneurial microvessels and in both myelinated and unmyelinated fibers, with a correlation between CML accumulation and myelinated fiber loss [142].

Furthermore, aminoguanidine treatment reduced experimental diabetic neuropathy, as shown by a significant improvement of the reduced motor nerve conduction velocity, partially through the correction of the endoneurial microcirculation [141].

Taking all this together, considerable evidence exists supporting that AGE contribute prominently to the development and exacerbation of diabetic neuropathy.

AGE in Renal Insufficiency, Renal Replacement Therapy and Kidney Transplantation

Accumulation of reactive carbonyl compounds (RCO) and AGE is markedly increased in plasma, serum and tissues of diabetic and non-diabetic patients with ESRD [143-145]. This has also been shown by Meng et al. for CML levels in plasma and skin in patients with chronic renal failure (CRF) both with and without diabetes [146]. Thus, hyperglycaemia is not a prerequisite for AGE accumulation. High serum AGE levels are also found in patients on hemodialysis (HD) or continuous ambulatory peritoneal dialysis (CAPD), probably by the introduction of high concentrations of glucose in dialysis fluids in CAPD (74-214 mM) [3,147]. Impaired clearance of AGE and AGE precursors is responsible for this increased AGE accumulation. Although direct evidence is insufficient, increased oxidizing conditions and the development of reactive oxygen species might also accelerate the formation of AGE [8,148,149]. Polymorphonuclear leukocyte activation by uremic toxins and interaction with the dialysis membrane in HD facilitate this. Diminished clearance of low molecular weight precursors of AGE and diminished catabolism of AGE precursors may further contribute to tissue AGE accumulation in CRF [3,150]. Lal et al. addressed the question of AGE-dependent signaling in the mesangium and found evidence for an important role of oxidative stress [149]. HD clears only the free and not the protein bound forms of AGE. CAPD is associated with lower plasma pentosidine levels, possibly by clearance of albumin-bound pentosidine in the peritoneum [3]. In rats
structural and permeability changes of the peritoneum with increased nitric oxide synthase expression are found associated with vascular deposits of AGE in chronic uraemia [151]. As for the relation with cardiovascular complications in renal failure, remarkably Schwedler et al. showed that circulating AGE levels (total fluorescent AGE, and CML, both in serum) do not predict mortality in HD patients [152]. Plasma pentosidinme as an AGE and marker of carbonyl stress, was neither found to be related to intima media thickness or atherosclerotic plaques in the carotids in HD patients [153]. Schwedler et al. proposed that the benefit of high serum AGE levels might reflect a better nutritional support, which improves survival. However, an alternative explanation is that plasma or serum AGE levels may not adequately reflect tissue AGE accumulation. Floridi et al. found that a shift from standard dialysis regimen 3 times a week to daily HD resulted after 6 months in substantially lower plasma AGE levels (pentosidine and two AGE peptides), but did not measure AGE in other tissues [154]. High concentrations of glucose (and alpha-oxoaldehydes) in dialysis fluids also increase glycation. Vitamin-E coated dialysis filters prevented the rise in serum AGE levels found with conventional polysulfone filters. After six months of HD with these vitamin E coated filters, the pre-dialysis levels of AGE and other markers of oxidative stress were reduced [155]. Very high flux polysulfone filters reduced pre-dialysis serum values of free and protein-bound pentosidine and AGE peptides and beta2-microglobulin (B2-MG) [156]. Several different AGE have been detected in B2-MG amyloid deposits arising after chronic renal HD [157]. Amyloid deposits are found in bone, articular cartilage, synovium, muscle and ligaments, giving rise to shoulder periarthritis, carpal tunnel syndrome and flexor tendosynovitis. AGE-modified B2-MG induces chemotaxis of monocytes and the production of cytokines (interleukin (IL) 1b, IL-6 and TNFa). AGE-modified B2-MG is cleared less than native B2-MG through dialysis membranes [157]. Hou et al. showed that the local inflammatory response to B2-MG amyloid deposits by monocytes/macrophages which is a characteristic histological feature of dialysis-related amyloidosis (DRA), is dependent on delayed apoptosis of monocytes by AGE-modified B2-MG [76]. These cytokines may stimulate local production of B2-MG and bone destruction. RAGE expressed by synovial
fibroblasts may mediate some of these responses involved in DRA [158]. CML-haemoglobin has been proposed as a possible predictor of progression of bone cysts in DRA [159]. Thus, at least a close relation between AGE accumulation in renal failure and the development of dialysis related amyloidosis exists. As for the possible osseous sequela of AGE accumulation, it should be added that pentosidine is related inversely to parathyroid hormone (PTH) and bone alkaline phosphatase in HD patients. The authors found this to be in agreement with AGE accumulation as a factor in reduced bone turnover in dialysis patients [160]. Yamamoto also found that AGE-BSA in vitro inhibits osteoblastic activity and inhibits PTH secretion in response to hypocalcaemia [161].

Renal transplantation is considered to be the best therapy for lowering AGE in patients with renal failure. Although a dramatic reduction in plasma AGE levels following transplantation has been found, the levels remain higher compared with the general population [162]. Studies to date, investigating the influence of kidney transplantation on tissue AGE accumulation show contradictory results [36,163-165]. While most studies report a reduction of about 50%, the only prospective study showed no significant reduction in tissue AGE. Despite the improvements in immunosuppression and an excellent short-term transplant survival in kidney transplantation, 10-year graft survival remains relatively poor with 50-60% (chronic renal transplant dysfunction, CRTD) while cardiovascular morbidity and mortality also remain high, and are associated with an increased prevalence of cardiovascular risk factors. Perhaps persistently high levels of AGE in tissues, enhanced by sustained oxidative stress, contribute to the development of CRTD. When AGE are administered to healthy rats, CRTD-like lesions develop in the kidney [20]. At present, no data is available on the long-term course of plasma and tissue AGE in human CRTD.

AGE ACCUMULATION IN OTHER DISEASES

Atherosclerosis

The accumulation of AGE in the vessel wall has also been related to lipoprotein metabolism, as discussed above, and to the development of atherosclerosis [166]. AGE
accumulation has been found by immunohistochemistry on the extracellular matrix proteins and within macrophage- and smooth muscle cell-derived foam cells in atherosclerotic plaques [114,167]. Normolipidemic and euglycaemic subjects with atherosclerosis had increased AGE in the apolipoprotein B100, as did patients with uraemia [1,118]. It was claimed that AGE-modified LDL had decreased clearance from circulation and metabolism but this was a characteristic of LDL glycated to higher extent than found physiologically [168]. The expression of the RAGE and scavenger receptors were increased in atherosclerosis [169]. Interaction of AGE-modified proteins with AGE receptors may stimulate cytokine and growth factor production that sustains the development of the atherosclerotic plaques [109,170-172]. Atherosclerotic lesions in apoE knockout mice that were accelerated under STZ-induced diabetic conditions were significantly suppressed by intraperitoneal administration of a soluble form of RAGE [120]. The process of atherosclerosis may also stimulate local AGE formation. Activated macrophages in the intima make the local environment of atheroma oxidizing, which may increase AGE formation. If glycation of LDL stimulated its oxidation in vivo then glycation would be a critical initiating factor in macrovascular disease. Currently, however, there is no agreement on this, although glycated collagen stimulated peroxidation of membrane lipids in vitro [173,174]. Other factors influence LDL oxidation - antioxidant and triglyceride content, for example - and it is not yet clear how important glycation is compared to these. Once atherosclerosis is initiated, however, increased formation of AGE within the locus of the plaque and induction of AGE receptor expression is expected to sustain plaque expansion.

Alzheimer’s Disease (AD)

AGE accumulation is found in senile plaques and neurofibrillary tangles of persons with AD [175-177]. In the cerebrospinal fluid of patients with AD increased levels of Amadori products on all the major proteins are found. This may contribute to increased AGE deposition in the brain of AD patients [178]. Although Meli et al. reported increased serum pentosidine levels, using HPLC, in patients with AD, so far no other
Clinical relevance of AGEs

evidence exists for systemic increases of AGE levels [179]. In contrast, chromatographic analysis of acid hydrolysates of brain frontal cortex for CML and pentosidine showed no significant increase in AD subjects, relative to controls. Pyrraline and pentosidine were not detected in beta-amyloid or apolipoprotein-E extracted from brain tissue of AD subjects, and immunohistochemistry with an anti-AGE antibody showed no staining of senile plaques [180,181]. Data on RAGE expression and binding in AD are also controversial. Thus, the role of AGE in AD is, therefore, currently uncertain. It has been proposed that reactive carbonyls are implicated as neurotoxic mediators of oxidative damage in the progression of AD and so may result in AGE accumulation [182].

Amyotrophic Lateral Sclerosis (ALS)

In sporadic ALS, CML as a marker of lipid peroxidation or protein glycoxidation and pentosidine as a marker of protein glycoxidation are increased along with markers of lipid peroxidation immunohistochemically in the spinal cord in almost all motor neurons and in reactive astrocytes and microglia/macrophages [183]. The authors implicate the formation of CML, pentosidine or lipid peroxidation products in motor neuron degeneration. A special familial form of ALS exists with superoxide dismutase-1 (SOD-1) gene mutations, characterized by the presence of neuronal Lewy body-like hyaline and astrocytic hyaline inclusions. Ultrastructurally these inclusions contain granule-coated fibrils positive for a SOD-1 protein but also modified by AGE. The formation of AGE-modified mutant SOD-1 is probably one of the mechanisms responsible for the fibril formation [184].

Arthritis

Drinda et al. detected CML in the synovial tissue in patients with osteoarthritis and also rheumatoid arthritis [185]. Adverse changes induced by AGE in the collagen network in bone are associated with a loss of toughness of bone in elderly people [186]. De Groot et al. found in in vitro experiments with cartilage from osteoarthritis or rheumatoid arthritis patients that increased cartilage AGE reduced cartilage
degradation by matrix metalloproteinases (MMP) in synovial fluid, suggesting that aged cartilage is less sensitive to the MMP-mediated cartilage destruction that occurs in osteoarthritis and rheumatoid arthritis. This may have effect on their rate of progression [187].

Amyloidosis

In various types of amyloidosis abnormal amyloid fibril proteins are associated with AGE accumulation [188]. Familial amyloidotic polyneuropathy (FAP), Portuguese type, is caused by amyloid depositions of a mutated transthyretin, and is associated with gastrointestinal symptoms like diarrhea, malabsorption due to severe dysmotility of the gastrointestinal tract. RAGE has been implicated in amyloid toxicity, and transthyretin amyloid fibrils have been shown to have affinity for RAGE and to induce NF-kappaB activation and apoptosis (also see above for RAGE effects). In autopsy samples immunoreactivity for AGE and RAGE was found at the same sites as amyloid deposits and transthyretin [189,190].

Periodontitis

AGE present in diabetic gingiva may be associated with a state of enhanced oxidant stress, a potential mechanism for accelerated tissue injury [191]. The degree of collagen crosslinking has a marked influence on bone regeneration of calvarial defects in rat studies. This may suggest a negative role of AGE-crosslinked collagen on recovery from periodontitis [192].

INTERVENTIONS

Different approaches may be used to counter AGE accumulation and its deleterious effects. The first approach is to reduce the formation of AGE at one of the many steps involved. This ranges from interventions aimed at reducing glucose levels in diabetes to drugs specifically aimed at one of the late steps in the Amadori process. Recent studies of the group of Vlassara et al. address the possibility to reduce AGE accumulation by limiting the exogenous supply of AGE from food. Since smoking is
Clinical relevance of AGEs

Another source of exogenous AGE and associated with increased AGE accumulation, therefore smoking cessation is another candidate in this category. The second approach is to increase breakdown of AGE. One drug, ALT-711, is now in advanced phase II studies as an AGE-breaker. The third approach aims to prevent the deleterious effects of AGE. It includes interventions aimed at reducing protein cross-links, or at blocking or competing for one or more of the different AGE receptors (summarized in Table 3).

Table 3. Interventions to reduce AGE accumulation.

<table>
<thead>
<tr>
<th>Inhibition of AGE formation</th>
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<tbody>
<tr>
<td>Specific: aminoguanidine, OPB-9195, ALT-946, pyridoxamine (or other “Amadorins”)</td>
</tr>
<tr>
<td>Aspecific: metformine, cerivastatine, ACE-inhibitors, ATII-antagonists, other pharmaceuticals that reduce oxidative stress</td>
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<table>
<thead>
<tr>
<th>AGE breaker</th>
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<td>ALT-711</td>
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<table>
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<tr>
<th>AGE-RAGE interaction blockers</th>
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<table>
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<tr>
<th>Reduction of exogenous AGE</th>
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<tr>
<td>ceasing tobacco use, low-AGE diet</td>
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</tbody>
</table>

Inhibitors of AGE Formation

As discussed above AGE formation is a complex process and under the influence of diverse mechanisms like glycation and oxidative stress. This offers many opportunities to prevent AGE formation. In fact, any treatment aimed at lowering glycation or oxidative stress may be considered. Usually, however, attention is focused more on interventions aimed at one of the steps in the Amadori process directly preceding AGE formation. The drug attracting most attention has been aminoguanidine.

Aminoguanidine. In 1986 Brownlee et al. showed that aminoguanidine prevented both the formation of fluorescent AGE and the formation of glucose-derived collagen cross-links in vitro [193]. Furthermore, administration of the drug to diabetic rats prevented fluorescent AGE-formation and cross-linking of arterial wall connective tissue protein in vivo. Aminoguanidine reacts with Amadori-derived reactive intermediates such as 3-deoxyglucosone. Incubation of model Amadori products with aminoguanidine produces a triazine compound which prevents the further rearrangement of intermediates to protein-protein and protein-lipid crosslinks [194]. Price et al. addressed the chelating activity of aminoguanidine and other AGE inhibitors (carnosine, OPB-9195) and breakers (ALT-711). They conclude that rather
than by trapping reactive carbonyl intermediates they act primarily as chelators and antioxidants [195]. Aminoguanidine does not interfere with already formed AGE. Aminoguanidine also reduces NO formation through inhibition of inducible NO-synthase. Several studies claim that aminoguanidine selectively inhibits NO-synthase, probably both the constitutive and the inducible form [196-200]. Furthermore, aminoguanidine may act as a free radical scavenger and so reduce oxidative stress [201,202]. Many studies have addressed its use in preventing diabetic micro- and macrovascular complications. The beneficial effect of aminoguanidine is considered by most authors to be mainly due to its direct effect on AGE formation. However, dissociations between reductions in albuminuria and effects on skin AGE formation have also been described [203]. Studies in diabetic animal show that aminoguanidine treatment prevents or reduces the major long-term complications of diabetes, nephropathy, neuropathy and retinopathy. Reduction of AGE accumulation, albuminuria and kidney lesions were established [90,136,204,205]. It is remarkable that the lesions at the tubulointerstitial level are improved by aminoguanidine [206,207]. Increase in motor nerve conduction velocity, partially through correction of endoneurial microcirculation, normalization of nerve blood flow and improvement of nerve conduction were also found [141,208,209]. Diabetic retinopathy was inhibited by aminoguanidine treatment, with prevention of abnormal endothelial cell proliferation and microaneurysms, and reduction in pericyte dropout, even when in one of these studies no effect of aminoguanidine on AGE (pentosidine in aorta and tail collagen, Hb-AGE) [122,209]. Clinical studies with aminoguanidine or its generic form, pimagedine, are still in progress.

In addition, aminoguanidine interferes with the development of atherosclerotic lesions: increasing elasticity and decreasing fluid filtration in large arteries, decreasing levels of total cholesterol, triglycerides and LDL-cholesterol in diabetic patients treated with aminoguanidine [210].

Outside the field of diabetes aminoguanidine reduced aortic pulse wave velocity in non-diabetic rats. Aminoguanidine seems to prevent the age-related increase in wall stiffness, although no effects on wall stress or composition were found [211].
Clinical relevance of AGEs

As for toxicity, a small percentage of patients develops flu-like symptoms. During longer use, macrocytic anaemia is found in a considerable percentage of patients and antinuclear cytoplasmic antibodies may develop at high doses, which is clinically associated with crescentic glomerulonephritis in three cases. Further side effects in human trials included nausea and headache. The daily dosage of aminoguanidine is usually 300 mg a day, taken with food. Aminoguanidine has a half life of only 4-hours, so these dosages would be best administered over the day into split doses. Checks of full blood counts and autoimmune antibodies are advised before and during treatment.

Other aminoguanidine-like compounds, without an influence on NO-synthase, have recently been described. They exhibit similar effects as aminoguanidine: 2,3 dianinophenazine (NNC39-0028) attenuated the development of diabetic mesenteric hypertrophy [212]; Oturai et al. found in STZ diabetic rats improved collagen solubility, in contrast to the lack of effect of the AGE-breaker PTB (see below). However, the increased urinary albumin excretion and the increased leakage in the eye of labeled albumin were not reduced by either drug [213]. 4-amino-3-hydrazone-5-isopropyl 4H-1,2,4-triazole and 3,5-diamino-4-hydroxy-benzoic acid dihydrochloride increased tail tendon collagen solubility in treated rats [105]. OPB-9195 [(+/-)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-yla cetanilide] inhibits both AGE and advanced lipoxidation endproducts (ALE) formation probably by its ability to trap reactive carbonyl compounds like GO, MGO and 3-DG [214]. It improved the delayed motor nerve conduction velocity in diabetic rats, and reduced the expression of immunoreactive AGE in the nerves [215]. ALT-946 was more potent than aminoguanidine in diabetic rats in suppressing immunohistochemically measured AGE production after 32 weeks. Albuminuria was equally reduced in the three groups treated from week 0 to 32 with aminoguanidine, or from week—32 or 16-32 with ALT-946 [216]. In a transgenic rat model with angiotensin II dependent hypertension, along with STZ-induced diabetes and severe diabetic nephropathy ALT-946 and aminoguanidine both reduced glomerulosclerosis and glomerular AGE immunolabeling. ALT-946 but not AG also reduced cortical tubular degeneration and albuminuria. Thus even with a strong hypertensive stimulus for developing
nephropathy in diabetic rats AGE formation inhibitors may protect against diabetic nephropathy [217].

Pyridoxamine inhibits formation of AGE derived from the alpha-dicarbonyl compound MGO in vitro [218]. It also reduces formation of lipoxidation end products (ALE). It has this capacity at least in part by trapping reactive carbonyl intermediates in the formation of AGE and ALE [219]. In diabetic rats pyridoxamine prevented after 29 weeks the development of structural changes of diabetic retinopathy and CML accumulation in the retinal vasculature [220]. Diabetic nephropathy is also inhibited by pyridoxamine to a similar extent as aminoguanidine in STZ-diabetic rats, associated with a marked reduction of AGE/ALE, CML, CEL, cross-linking and fluorescence in skin collagen [221].

In 1999, a novel group of inhibitors of the conversion of Amadori intermediates to AGE, named "Amadorins" was described [222]. The therapeutic potential of these drugs is currently being investigated. Other agents such as Carnosine and Acetyl-L-Carnitine also prevent AGE formation. Carnosine (beta-alanyl-lhistidine) is physiologically present in muscle and nervous tissues in many animals, especially long-lived species [223]. As yet only in vitro studies found that N-acetylcysteine suppressed CML production during prolonged incubations [224].

In a recent study it was demonstrated that ACE inhibition with ramipril and aminoguanidine after 12 weeks equally reduced renal AGE accumulation, assessed by immunohistochemistry and serum and renal fluorescence, in STZ-induced diabetic animals. Both drugs prevented diabetes-induced increases in expression of RAGE and also AGE R2, R3 receptors and NFkappaB. The link between inhibition of the renin-angiotensin system and of AGE accumulation may reside in the reduction of oxidative stress [225]. The inhibition of AGE production by interventions in the renin-angiotensin system is confirmed by an in vitro study by Miyata et al. for the angiotensin II type 1 receptor antagonist olmesartan and the ACE-inhibitor temocaprilat. In a comparison with nifedipine, both olmesartan and temocaprilat inhibited the formation of pentosidine and CML [226]. An in vitro study with different
calcium antagonists showed some antioxidative properties of calcium antagonists but no inhibitory effect on the formation of AGE precursors [227].

Beraprost sodium, a prostacyclin analogue and cyclic-AMP-elevating agent, was found to protect retinal pericytes with prevention of AGE-induced apoptotic cell death from AGE-induced cytotoxicity through its antioxidative properties [228].

**AGE-Breakers**

In 1996, Vasan and colleagues described a new compound that cleaves glucose-derived protein cross-links in vitro and in vivo [229]. Its action is based on the assumption that AGE are formed in a pathway involving reactive α-dicarbonyl intermediates. The AGE-breakers Nphenacythiazolium bromide (PTB) or the chloride form developed by Alteon ALT-711, 3-phenacyl-4, 5-dimethylthiazolium chloride, is able to break this α-dicarboxyl bond, and can thereby remove established AGECrosslinks (Table 3). However, taking into account the high reactivity of these α-dicarboxyl bonds, a crosslink containing this bond is not likely to occur. Because in vitro and in vivo studies indeed confirm the ability of PTB to actually diminish AGE derived crosslinks, therefore, future research should be focused on finding different mechanisms by which PTB breaks AGE crosslinks. Wolffenbuttel et al. performed the first in vivo study in rats, showing that chronic treatment with an AGE-breaker restored large artery properties in experimental diabetes [230]. In a study in aging dogs ALT-711 1mg/kg reversed the age-related increased stiffness of the myocardium within four weeks [231]. Left ventricular stiffness was reduced by approximately 40%. Studies on the effects of ALT-711 on older rhesus monkeys showed that an injection of 10 mg/kg ALT-711, every other day for three weeks, improved vascular compliance and elasticity [232]. The improved vascular compliance persisted over time, with maximum improvement seen six weeks after the end of ALT-711 treatment, and a gradual return to baseline 39 weeks after treatment was stopped.

After phase I clinical studies with ALT-711, Kass et al. described last year a randomized, double blind, placebo controlled trial in 93 individuals over the age of 50, with as selection criterion a systolic blood pressure > 140 mmHg and pulse pressure of
at least 60 mmHg [233]. Treatment consisted of ALT-711 210 mg daily or placebo for eight weeks, in addition to usual antihypertensive treatment. ALT-711 lowered pulse pressure with 5.6 mmHg drops in the treated group compared to a -0.5 mmHg drop in patients receiving placebo. ALT-711 significantly improved large artery compliance and distensibility by 11% to 18%, compared to placebo. The drug is now tested in several phase II trials in isolated systolic hypertension, addressing the vascular and left ventricular effects (Alteon website: SAPPHIRE and SILVER trials) and in diastolic heart failure.

**AGE-RAGE Interaction Blockers**

As discussed above, AGE-RAGE interactions may be important in numerous diseases. Attempts to block the deleterious effects of AGE at the receptor level are still in an experimental phase [74].

**Prevention of other AGE-Induced effects**

A novel approach in preventing deleterious effects of AGE has been proposed by Okamoto. He used incadronate to prevent AGE-induced angiogenesis from microvascular endothelial cells probably by blocking protein farnesylation [234].

Cerivastatin, a lipid lowering drug from the class of statins, prevents the AGE-induced increase in NF-kappaB and activator protein-1 activity, suppressed VEGF mRNA upregulation in cultured endothelial cells [235].

**Interventions Aimed at Reducing Exogenous AGE, Food and Smoking**

Vlassara addressed the effect of food derived proinflammatory AGE on inflammatory mediators in diabetic subjects by comparing otherwise comparable diets with a high AGE content or with a 5-fold lower AGE content. Serum AGE, measured as CML-sensitive ELISA, were approximately 70% higher on the high AGE compared to the low AGE diet, AGE-LDL was approximately 40% higher. The levels of the endothelial adhesion molecule VCAM-1 and mononuclear tumor necrosis factor (TNF) alpha were considerably higher during the high AGE diet [236]. Although the study was too small
to draw definite conclusions, the results suggest that high AGE diets may result in higher levels of inflammatory mediators in diabetes patients and so contribute to the chronic complications. In apoE-deficient mice in whom a femoral artery injury had been induced, a low AGE diet compared to a diet with a tenfold higher AGE content resulted, 4 weeks after the injury in a decrease in neointima formation and a less stenotic luminal area [237]. Markedly lower number of macrophages and a reduced number of smooth muscle cells, associated with a 40% decrease in circulating AGE levels and AGE deposition in endothelial cells, smooth muscle cells and macrophages of the neointima in the low AGE diet mice were observed. The same group tested the effects of a low AGE diet also in two diabetic mouse models for type 1 and type 2 diabetes, respectively. The mice on a low AGE diet had a marked reduction in the diabetic nephropathy glomerular lesions and an extended survival [238]. In the renal cortex expression of transforming growth factor-beta1, laminin B1 mRNA and alpha-IV collagen mRNA was lower in the low AGE diet group, along with reduced serum and kidney AGE levels. The relation between diet and exogenous AGE load and resulting (serum) AGE levels is not always straightforward: Sebekova expected to find higher AGE levels in omnivores eating more heat-treated food would be higher than in vegetarians cooking their food. However, plasma AGE levels (measured with ELISA for CML, also plasma fluorescence) were higher in the vegetarians [239]. More specific food substances, for example green tea extracts, have been tested for their effect on protein glycation and oxidation in vitro [240]. Gugliucci found that in vitro extracts of the flavonoid-rich herbal species Achyrocline satureoides and Ilex paraguariensis inhibited the effects of the reactive carbonyl compound methylglyoxal on heparin activation of antithrombin III and plasminogen activity [241].

CONCLUSIONS
AGE have evolved from noxious bystanders of long-time glycation to endproducts of both oxidative and carbonyl stress. In association with the expansion in the biological effects of AGE, this makes AGE the primary targets for pharmaceutical interventions.
Pharmaceutical strategies can be aimed both at prevention of AGE formation and at AGE breaking. A weak point remains the dearth of feasible AGE assay methods available for clinical practice to monitor the effects of such pharmaceutical interventions. Introduction of reproducible, easily available assays of tissue AGE accumulation are warranted for a more focused application of treatment strategies.

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