# Diabetes, Non-Enzymatic Glycation, and Aging

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## Introduction

Societal obsession with the process of aging dates back to ancient history, and myths related to the conservation of youth—ranging from a bathing fountain that confers eternal youth to a philosopher's stone that could be used to create an elixir of life—populate both past and contemporary folklore. However, it is only within recent years that aging has been investigated from an empirical approach, as it continues to garner increasing attention from the scientific community. While several hypotheses have been proposed to explain the pathophysiology responsible for senescence, no single theory accounts for the diverse phenomena observed. Rather, aging appears to be a multifactorial process that results from a complex interplay of several factors and mechanisms [1].

Aging is characterized by a decline of anatomical integrity and function across multiple organ systems and a reduced ability to respond to stress. The multisystem decline is associated with increasing pathology, disease, and progressively higher risk of death. Although the true mechanisms that drive the aging process are still a

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mystery, there is evidence that both genetic and environmental factors may affect the rate of appearance of phenotypes characteristic of the aging process. Thus, aging appears in part to be modulated by a genetic–environmental interaction [2]. Studies of gene expression across species and tissues have consistently observed that old age is associated with progressive impairment of mitochondrial function, increased oxidative stress (OS), and immune activation [3]. Interestingly, all these processes can be influenced by modification of nutritional intake. For example, studies of animal species have found that caloric restriction reduces OS and is associated with longer life expectancy. Recent studies have cast doubts on whether humans may be able to maintain a long-term regimen of caloric restriction without unacceptable psychological consequences and whether even caloric restriction may have overall positive health effects in humans [3]. It has been suggested that changes in the quality of the diet could have positive effects on health and longevity and could be more easily implemented compared with caloric restriction.

Nevertheless, stratification of factors and mechanisms contributing to senescence is critical for the development of initial strategies in combating the aging process. The skin is an excellent paradigm for studying aging, in large part due to its easy accessibility. Moreover, in addition to its vulnerability to internal aging processes because of its diverse role in cellular processes, such as metabolism and immunity, the skin is subject to a variety of external stressors as the chief barrier between the body and the environment.

Aging factors can generally be classified as exogenous or endogenous. As ultraviolet (UV) radiation exposure is so strongly associated with a host of age-related skin diseases, endogenous and exogenous factors can theoretically be studied somewhat independently in the skin by differentiating between UVprotected and UV-exposed sites [4]. Endogenously aged skin displays characteristic morphological features with resultant alterations in functionality. These include epidermal, dermal, and extracellular matrix (ECM) atrophy leading to increased fragility, diminished collagen and elastin resulting in fine wrinkle formation, and marked vascular changes disrupting thermoregulation and nutrient supply. Endogenously aged skin also displays decreased mitotic activity, resulting in delayed wound healing, as well as decreased glandular function, resulting in disturbed reepithelialization of deep cutaneous wounds. Also seen is a reduction of melanocytes and Langerhans cells manifesting as hair graying and higher rates of infection, respectively [4–13].

Exogenously aged skin, in which environmental factors such as UV radiation act in concert with endogenous processes, shares many of the characteristics of endogenously aged skin. In addition, exogenously aged skin displays a thickened epidermis and aggregation of abnormal elastic fibers in the dermis (i.e. solar elastosis) [4].

Among the many mechanisms thought to underlie aging, glycation has emerged in recent years as one of the most widely studied processes. Testament to the rapidly growing attention from the scientific community, a cursory literature search will yield thousands of articles related to glycation, the majority of them published in the last decade. *Glycation refers to the non-enzymatic process of proteins, lipids, or nucleic acids covalently bonding to sugar molecules, usually glucose or fructose.* The lack of enzyme mediation is the key differentiator between glycation and

glycosylation. Glycosylation occurs at defined sites on the target molecule and is usually critical to the target molecule's function. In contrast, glycation appears to occur at random molecular sites and generally results in the inhibition of the target molecule's ability to function. The products of glycation are called advanced glycation end products.

AGEs were first identified in cooked food as end-products from a non enzymatic reaction between sugars and proteins called the Maillard reaction [14]. The Maillard reaction (non enzymatic glycation (NEG) or browning) in foods has been well studied by the food industry to control food quality. However, it is only 40 years ago that a similar glycation process was recognized in human body by the observation of increased formation of glycosylated haemoglobins in diabetic patients [15] and this would lead to the formation of detrimental AGEs in humans [16].

The body also produces AGEs naturally as it processes sugars. The formation of AGEs is a part of normal metabolism, but if excessively high levels of AGEs are reached in tissues and the circulation they can become pathogenic [17].

The pathologic effects of AGEs are related to their ability to promote OS and inflammation by binding with cell surface receptors or cross-linking with body proteins, altering their structure and function [18]. The formation and accumulation of AGEs is a characteristic feature of tissues in aged people, especially in patients with Diabetes Mellitus (DM), and these products have also been strongly implicated in the pathogenesis of age-related and diabetic complications. Many chronic diseases, including heart diseases, Diabetes and both osteoarthritis (OA) and rheumatoid arthritis are associated with inflammation. It has been reported that AGEs are involved in musculoskeletal diseases such as OA, which is the most common chronic disabling disorder for aged people. Accumulation of AGEs increases stiffness of the collagen network in the bone as well, which may explain some of the age-related increase in skeletal fragility and fracture risk [19]. AGEs can be particularly dangerous for diabetics, as the increased availability of glucose in Diabetes patients accelerates the formation of AGEs.

AGEs are a heterogeneous group of macromolecules that are formed by the NEG of proteins, lipids, and nucleic acids. Humans are exposed to two main sources of AGEs: exogenous AGEs such as tobacco and certain foods [20, 21] and endogenous AGEs that are formed in the body. The Western diet is rich in AGEs. AGEs are formed when food is processed at elevated temperatures, such as during deep-frying, broiling, roasting, grilling; high temperature processing for certain processed foods such as pasteurized dairy products, cheeses, sausages, and processed meats; and commercial breakfast cereals. Endogenous AGEs are generated at higher rates in diabetics due to altered glucose metabolism. AGEs, by increasing OS and through other mechanisms, may accelerate the multisystem decline that occurs with aging and, therefore, reducing intake and circulating levels of AGEs may promote healthy aging and greater longevity [2].

Increased accumulation of AGEs was first directly correlated to the development of diabetic complications. Since then, AGEs have been implicated in a host of other pathologies, including atherosclerosis, end stage renal disease, and chronic obstructive pulmonary disease [22]. (It should be noted that AGE levels have been shown

to vary by race and gender, and until larger studies are done to create ethnic- and gender-specific reference values, increased accumulation of AGEs should be defined as levels that are elevated for all demographic groups) [23].

Not coincidentally, many of the pathologies associated with AGEs, including diabetic sequelae, are closely related to senescence.

This extends to aging skin, as methods of AGE detection, such as immunostaining, have demonstrated the prevalence of glycation in aged skin. Glycation results in characteristic structural, morphological, and functional changes in the skin, a process colloquially known as "*sugar sag*." With glucose and fructose playing such a prominent role in the mechanism, it is not surprising that diet plays a critical role in glycation and thus aging skin [1].

Perhaps more surprising, studies have shown that consumption of AGEs is not only tied to the sugar content of food, but is also affected by the method of cooking. Furthermore, as the connection between diet and aging is more clearly characterized, a host of dietary compounds have surfaced as potential therapeutic candidates in the inhibition of AGE-mediated changes [1].

Until today, more than 300 theories of aging have been proposed, among them the theory of cellular senescence, decreased proliferative capacity and telomere shortening, mitochondrial DNA single mutations, the free radical theory and others, none of which can fully explain all changes observed in aging [24–28]. Acording to the inflammatory theory of aging, a common characteristic of skin aging factors is their ability to induce or maintain proinflammatory changes and trigger a local inflammatory response which through subsequent immune responses, matrix metalloproteinase (MMP) activation and proinflammatory cytokine production contributes to the structural changes observed in aged skin [29].

In the recent years, the role of AGEs has been increasingly discussed in skin aging, and the potential of anti-AGE strategies has received high interest from pharmaceutical companies for the development of novel anti-aging cosmeceutical compounds [30]. The study of AGE represents one of the most promising areas of research today. Although the initial chemistry behind their formation has been known since the early 1900s, it is only in the last 20 years or so that important work has been done to elaborate on this. The chemical processes and pathways that ultimately lead to AGE formation have, however, yet to be fully clarified [31]. As our knowledge of AGE chemistry increases it is becoming apparent that not all AGE have been isolated, whereas as those that have been characterized are both complex and heterogenous. Thus, the discovery and investigation of AGE inhibitors would offer a potential therapeutic approach for the prevention of diabetic or other pathogenic complications [16].

## Biochemistry of AGEs

Glycation is the non-enzymatic reaction between reducing sugars, such as glucose, and proteins, lipids or nucleic acids [32]. Glycation has to be distinguished from glycosylation, which is an enzymatic reaction. Since its first description by Maillard

in 1912 and its involvement in food browning during thermal processing by Hodge 50 years later, its presence in living systems and involvement in various pathologies of the human body, including aging and Diabetes, have been an intensive field of research [33, 34].

First described over a century ago, glycation entails a series of simple and complex non-enzymatic reactions. In the key step, known as the Maillard reaction, electrophilic carbonyl groups of the sugar molecule react with free amino groups of amino acids (especially of basic lysine or arginine residues), leading to the formation of a non-stable Schiff base [35]. This non-stable Schiff base contains a carbon-nitrogen double bond, with the nitrogen atom connected to an aryl or alkyl group. The Schiff base rapidly undergoes re-arrangement to form a more stable ketoamine, termed the Amadori product [32, 35]. Schiff bases and Amadori products are reversible reaction products. At this juncture, the Amadori product can: (1) undergo the reverse reaction; (2) react irreversibly with lysine or arginine functional groups to produce stable AGEs in the form of protein adducts or protein cross-links; or (3) undergo further breakdown reactions, such as oxidation, dehydration, and polymerization, to give rise to numerous other AGEs [32, 36]. AGE formation is accelerated by an increased rate of protein turnover, hyperglycemia, temperatures above 120°C (248°F), and the presence of oxygen, reactive oxygen species (ROS), or redox active transition metals [36]. When an oxidative step is involved, the products are called advanced glycoxidation end products [32, 36].

The first, and perhaps most well-known, physiological AGE to be described was glycated hemoglobin (HbA1c), now widely used to measure glycemic control in Diabetes.

*Since the discovery of HbA1c in Diabetes, numerous other AGEs have been detected. Some of them have characteristic autofluorescent properties, which simplifies their identification in situ or in vivo* [32, 36–47].

However, the most prevalent AGE in the human body, including the skin, is carboxymethyl-lysine (CML), first described by Ahmed which is formed by oxidative degeneration of Amadori products or by direct addition of glyoxal to lysine. It seems to be the major epitope of the commonly used polyclonal anti-AGE antibodies [48].

It is a non-fluorescent protein adduct. In the skin, CML is found in the normal epidermis, aged and diabetic dermis, and photoaging-actinic elastosis [37–39].

Other AGEs detected in skin include pentosidine, glyoxal, methylglyoxal, glucosepane, fructose lysine, carboxyethyl-lysine, glyoxal-lysine dimer, and methylglyoxal-lysine dimer [35, 49].

Pentosidine was first isolated and characterized by Sell and Monnier. It is composed of an arginine and a lysine residue crosslinked to a pentose [50]. Pentosidine is a fluorescent glycoxidation product and forms protein-protein crosslinks [35].

Dicarbonyl compounds like 3-deoxyglucosome, methylglyoxal and glyoxal derive from oxidative degradation or autooxidation of Amadori products and other pathways [32, 51]. These dicarbonyl compounds are very reactive molecules leading to protein crosslinks [32].

*Since the discovery of the first glycated protein, glycated hemoglobin in diabetes, numerous other AGEs have been detected. Some of them have characteristic*

*autofluorescent properties, which simplifies their identification in situ or in vivo [32]. To date, numerous AGEs have been identified [36–47].*

Environmental factors, such as diet and smoking influence the rate of AGE formation [52, 53]. Moreover, it seems that the level of circulating AGEs levels are genetically determined, as shown in a cohort study of healthy monozygotic and heterozygotic twins [54].

The content of AGEs in the organism is not only defined by the rate of their formation but also by the rate of their removal. Many cells have developed intrinsic detoxifying pathways against accumulation of AGEs [55]. The glutathione-dependent glyoxalase system, comprising of glyoxalase (Glo) I and II, has a key role in the defense against glycation [56]. This system uses reduced glutathione (GSH) to catalyze the conversion of glyoxal, methylglyoxal and other α-oxoaldehydes to the less toxic D-lactate [56]. Other enzymatic systems include fructosyl-amine oxidases (FAOXs) and fructosamine kinases, relatively new classes of enzymes which recognize and break Amadori products [57]. However, FAOXs or "amadoriases" have been found to be expressed only in bacteria, yeast and fungi but not in mammals.They oxidatively break Amadori products but act mostly on low molecular weight compounds [58]. On the contrary, fructosamine kinases are expressed in various genomes including humans [57]. These intracellular enzymes phosphorylate and destabilize Amadori products leading to their spontaneous breakdown [58]. Fructosamine-3-kinase (FN3K), one of the most studied enzymes in this system, is almost ubiquitary expressed in human tissues including the skin. Thus, it plays an important role in the intracellular breakdown of Amadori products [59].

## Receptors for AGEs

AGEs not only exert their deleterious actions due to their biological properties per se, but also through their interaction with specific receptors. Receptor for AGEs (RAGE) is a multiligand member of the immunoglobulin superfamily of cell surface receptors, encoded by a gene on chromosome six near the major histocompatibility complex III. It is a pattern recognition receptor binding in addition to AGEs various other molecules such as S-100/calgranulins, high motility group protein B1 (amphoterine), β-amyloid peptides and β-sheet fibrils [52, 60]. The binding of ligands to RAGE stimulates various signaling pathways including the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases (ERK) 1 and 2, phosphatidyl-inositol 3 kinase, p21Ras, stress-activated protein kinase/c-Jun-N-terminal kinase and the janus kinases [52, 60]. Stimulation of RAGE results in activation of the transcription factor nuclear factor kappa-B (NFκB) and subsequent transcription of many proinflammatory genes [60]. Interestingly, RAGE-induced NFκB activation is characterized by a sustained and self-perpetuating action, through induction of positive feedback loops and overwhelming of the autoregulatory negative feedback loops. RAGE activation leads to new synthesis of the transcriptionally active subunit p65, which overwhelms the newly synthesized inhibitor

IκBα. Moreover NFκB increases further expression of RAGE, which itself further stimulates NFκB, forming a vicious cycle of selfrenewing and perpetuating proinflammatory signals [60]. RAGE activation can directly induce oxidative stress by activating nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NOX), decreasing activity of superoxide dismutase (SOD), catalase and other pathways, and indirectly by reducing cellular antioxidant defenses, like GSH and ascorbic acid [60–62]. The reduction of GSH leads furthermore to decreased activity of Glo I, the major cellular defense system against methylglyoxal, therefore supporting further production of AGEs [38]. RAGE is almost ubiquitary expressed in the organism, typically at low levels, and its expression is upregulated under various pathologic conditions [60, 63]. In the skin, RAGE expression was observed in both epidermis and dermis, and it was increased in sun-exposed compared with UV irradiation-protected areas. Keratinocytes, fibroblasts, dendritic cells and to a lesser extent endothelial cells and lymphocytes express RAGE [63]. Not only in vivo, but also in vitro, various skin cells types have been shown to express RAGE [61, 63–68].

RAGE is the most studied receptor for advanced glycation end products. Another group of cell surface receptors, AGER1, AGER2 and AGER3 seem to regulate endocytosis and degradation of AGEs, thus counteracting the effects of RAGE [69]. AGER1 has been further shown to counteract AGEs-induced oxidative stress via inhibition of RAGE signaling [70, 71]. Soluble RAGE (sRAGE) is a truncated splice variant of RAGE containing the ligand-binding domain but not the transmembrane domain and has been found in plasma. sRAGE is a soluble extracellular protein without signaling properties and it is considered as a natural decoy receptor of RAGE [72].

## Toxicity of Advanced Glycation End-Products (AGEs)

The possible pathophysiological role of AGEs has become a topic of increasing interest over the past few years. With the continued research on the Maillard reaction, it was demonstrated that the Maillard reaction also occurs in vivo and the term *"glycation"* was introduced as a synonym for *"non-enzymatic glycosilation",* in order to distinguish this from the well known enzymatic glycosilation of proteins [73]. Protein modifications called *"Advanced glycation end-products"* (AGEs), which are formed during aging, Diabetes and in renal failure via comparable chemical pathways as described for heated foods, nowadays are generally accepted to play a pivotal pathophysiological role in several diseases [74]. In vitro studies using human derived endothelial cells exhibited the food-derived AGEs have same protein cross-linking and intracellular oxidant stress actions as their endogenous counterparts. In animal studies like in mice, reduction of dietary AGE intake is accompanied by significant reduction of circulating AGEs levels as well as reduction of diseases related to inflammation and oxidative stress. A low-AGE diet has been associated with a significant increase in mouse lifespan. The human relevance of the in vitro and animal data discussed in a number of studies found independent correlate of the circulating AGEs with the dietary AGEs intake. Moreover, the effect of a

low and a high-AGE diet on the inflammatory mediators was also studied by using a group of diabetic subjects. The low-AGE diet significantly reduced serum AGE levels as well as markers of inflammation and endothelial dysfunction. Thus, all these studies demonstrate the associated toxicity of AGEs [16, 75].

## AGEs and the Skin

AGEs accumulate in various tissues as a function, as well as a marker, of chronological age [76]. Proteins with slow turnover rates, such as collagen, are especially susceptible to modification by glycation. Collagen in the skin, in fact, has a half-life of approximately 15 years and thus can undergo up to a 50% increase in glycation over an individual's lifetime [77].

Collagen is critical not only to the mechanical framework of the skin but also to several cellular processes, and is impaired by glycation in multiple ways. First, intermolecular cross-linking modifies collagen's biomechanical properties, resulting in increased stiffness and vulnerability to mechanical stimuli [78]. Second, the formation of AGEs on collagen side chains alters the protein's charge and interferes with its active sites, thereby distorting the protein's ability to interact properly with surrounding cells and matrix proteins [79]. Third, the ability to convert L-arginine to nitric oxide, a critical cofactor in the crosslinking of collagen fibers, is impaired [80]. Finally, glycated collagen is highly resistant to degradation by matrix metalloproteinases (MMPs). This further retards the process of collagen turnover and replacement with functional proteins [81].

Other cutaneous extracellular matrix proteins are functionally affected by glycation, including elastin and fibronectin. This further compounds dermal dysfunction [49, 82] as glycation crosslinked collagen, elastin, and fibronectin cannot be repaired like their normal counterparts.

Interestingly, CML-modified elastin is mostly found in sites of solar elastosis and is nearly absent in sun-protected skin. This suggests that UV-radiation can mediate AGE formation in some capacity or, at the least, render cells more sensitive to external stimuli [83]. It is hypothesized that UV-radiation accomplishes this through the formation of ROS (superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals). This induces oxidative stress and accelerates the production of AGEs [84]. AGEs themselves are very reactive molecules and can act as electron donors in the formation of free radicals. Occurring in conjunction with the decline of the enzymatic system that eliminates free radicals during the aging process, these properties lead to a "*vicious cycle*" of AGE formation in the setting of UV exposure.

Formed both intracellularly and extracellularly, AGEs can also have an effect on intracellular molecular function. In the skin, the intermediate filaments of fibroblasts (vimentin) and keratinocytes (cytokeratin 10) have been shown to be susceptible to glycation modification [41]. Analogous to the diverse role of collagen in the skin, intermediate filaments are essential to both the maintenance of cytoskeletal stability and the coordination of numerous cellular functions. Fibroblasts with

glycated vimentin demonstrate a reduced contractile capacity, and these modified fibroblasts are found to accumulate in skin biopsies of aged donors [41].

In fact, general cellular function may be compromised in the presence of high concentrations of AGEs. In vitro, human dermal fibroblasts display higher rates of premature senescence and apoptosis, which likely explains the decreased collagen and extracellular matrix protein synthesis observed in both cell culture and aged skin biopsies [65, 85]. Similarly, keratinocytes exposed to AGEs express increased levels of pro-inflammatory mediators, suffer from decreased mobility, and also undergo premature senescence in the presence of AGEs [86].

In addition to intermediate filaments, proteasomal machinery and DNA can undergo glycation. Proteasomal machinery, which functions to remove altered intracellular proteins, decline functionally in vitro when treated with glyoxal [87]. Similar in vitro findings were observed when human epidermal keratinocytes and fibroblasts were treated with glyoxal, leading to accumulation of CML in histones, cleavage of DNA, and, ultimately, arrest of cellular growth [88].

Beyond the modification of host molecular physicochemistry, AGEs also exert detrimental effects through the binding to RAGE. As cited before, RAGE is a multiligand protein that, when activated, can trigger several cellular signaling pathways, that are known to mediate various pathogenic mechanisms through the alteration of cell cycle regulators, gene expression, inflammation, and extracellular protein synthesis [60]. Not surprisingly, RAGE is found to be highly expressed in the skin and is present at even higher levels in both UV-exposed anatomical sites and aged skin [63].

## Role of AGEs in the Skin Aging Process

Cutaneous accumulation of AGEs is a feature of skin aging. Accumulation of AGEs has been detected in various tissues during aging and Diabetes, including articular collagen, skeletal and smooth vascular muscles or glomerular basement membranes [89–91]. Accordingly, deposited AGEs in these tissues have been implicated in various Diabetes or age-associated pathologies such as diabetic angiopathy, age and diabetes-associated macular degeneration and osteoarthritis [81, 89–94].

Skin, due to its easy accessibility, offers an excellent opportunity for minimal invasive or even non-invasive investigation of glycation, taking advantage of the characteristic autofluorescent properties of AGEs. Accumulation of AGEs in the skin has been therefore thoroughly studied and is detected not only in Diabetes as expected but also during chronological aging [39, 95, 96]. Glycation associated skin autofluorescence was shown to correlate with chronological aging in a large number of healthy subjects [97].

It is a general perception today that AGE accumulation is dependent on protein turnover rate; therefore long-lived proteins are thought to be mainly modified by glycation [89]. Collagen types I and IV, exhibiting a slow turnover rate of about 10 year, and other dermal long-lived proteins like fibronectin mainly suffer from glycation during intrinsic chronological aging [38, 39]. The appearance of glycated

collagen is first observed at the age of 20. It accumulates with a yearly rate of about 3.7% reaching a 30–50% increase at 80 year of age [39, 77]. CML was recently histochemically detected in human epidermis from healthy donors [37]. The upper epidermal layers were mostly involved (stratum spinosum, granulosum and corneum) and the authors identified cytokeratin 10 (CK10) (expressed by differentiated keratinocytes) as a target protein for CML modification. The amount of CML in younger donors seemed to be weak in comparison to the older ones. The latter study had restrictions, as the size of the sample was small and heterogeneous, but indicates a potential involvement of AGEs in epidermal physiology and a possible involvement of more short-lived proteins in glycation chemistry. Moreover, in an in vitro reconstructed organ skin model, both epidermis and dermis, as well as their functions, were modified by glycation [98].

Moreover, smoking, a typical aggravating factor of skin aging, accelerates formation of AGEs and increases their deposition in various tissues including skin [82, 99]. Another important environmental factor for aging is diet. The content of AGEs in food is highly dependent on the method of preparation, like cooking time and temperature. Fried food contains in general far higher amounts of AGEs than boiled or steamed food [100]. Dietary AGEs directly correlate with serum levels of AGEs and inflammatory markers in healthy human subjects, respectively [101].

It has been widely accepted that AGEs, once formed, can be only removed when the modified proteins degrade. However it has now become apparent that in the organism various enzymatic systems seem to be involved in the degradation or removal of AGEs. As mentioned above, Glo I is an enzyme responsible for the removal of reactive α-dicarbonyl compounds. Interestingly, decreased activity of such defense systems against AGEs has been reported during aging [62]. These age-related changes may further increase the extent of deposited AGEs in a living organism over time.

Consequences of AGE deposition in skin. AGEs can be formed intracellularly and extracellularly. Their presence in biological molecules modifies their biomechanical and functional properties. Proteins, lipids and nucleic acids can be targets of advanced glycation, modifying enzyme-substrate interactions, protein-DNA interactions, protein-protein interactions, DNA regulation and epigenetic modulation, thus interfering with numerous physiological functions of the organism. Moreover, AGEs are themselves reactive molecules which through interaction with their receptors activate various molecular pathways in vivo, thus becoming involved in inflammation, immune response, cell proliferation and gene expression.

## Extracellular Matrix Proteins

ECM proteins have been regarded as one of the major target structures for glycation. The most abundant collagen type in the skin is type I, whereas collagen IV is being found in the basal membrane. Collagen is one of the strongest proteins. In the skin, it is not only used as a supportive framework for mechanical support for cells and

tissues, but represents an active component being able to interact with cells and affect various cellular functions such as migration, differentiation and proliferation.

Collagen glycation impairs its function in various ways. Intermolecular cross-links of adjacent collagen fibers change its biomechanical properties leading to stiffness and decreased flexibility, thus increasing its susceptibility to mechanical stimuli [78]. The change of its charge and the formation of AGEs on side chains of collagen affect its contact sites with cells and other matrix proteins and inhibit its ability to react with them [79]. The precise aggregation of monomers into the triple helix may be affected as well as the association of collagen IV with laminin in the basal membrane [35]. Modified collagen resists degradation by MMPs, thus inhibiting its removal and replacement by newly synthesized and functional one [81]. Accordingly, tissue permeability and turnover is impaired [35, 102].

Other ECM proteins suffering from advanced glycation are elastin and fibronectin, contributing further to dermal dysfunction [38, 39, 42]. Of note, CML-modified elastin has been found almost exclusively in sites of actinic elastosis and not in sun-protected skin, underlining its potential role in photoaging. Indeed, UV irradiation stimulates glycation of elastin in the presence of sugars. Moreover, CML-modified elastin assembled in large and irregular structures, has decreased elasticity and is resistant to proteolytic degradation [83].

It has been shown that in vitro glycated skin samples have impaired biomechanical properties [103]. In vivo, decreased skin elasticity characterizes diabetic subjects in comparison to healthy controls [104].

## Intracellular Proteins

Intermediate filaments such as vimentin in fibroblasts and CK10 in keratinocytes have been found to be modified by AGEs [37, 41]. Cytoskeletal proteins are important in providing stability of the cytoskeleton and are crucially involved in numerous cellular functions such as migration and cellular division. Various other intracellular proteins including enzymes and growth factors may be targets of NEG. Glycated basic fibroblast growth factor (bFGF) displays impaired mitogenic activity in endothelial cells [105]. Glycation of enzymes of the ubiquitin-proteasome system and of the lysosomal proteolytic system has been shown to inhibit their action [106]. Antioxidant and other protective enzymes such as Cu-Zn-SOD can be inactivated [107]. Other intracellular components, such as DNA and lipids can be glycated with detrimental effects on their function [32].

## Receptors for AGEs: RAGE

AGEs do not only act by altering the physicochemical properties of glycated proteins. As mentioned above, AGEs may bind to their cell surface receptor, RAGE, initiating a cascade of signals influencing cell cycle and proliferation, gene expression,

inflammation and extracellular matrix synthesis [60]. Interestingly, RAGE is broadly expressed in human skin and in epidermal keratinocytes, dermal fibroblasts and endothelial cells in vitro. It is highly found in sites of solar elastosis, and its expression is induced by AGEs and proinflammatory cytokines like TNFα [63]. In skin cells RAGE has been shown to decrease cell proliferation, induce apoptosis and increase MMPs production. Many of these effects involve NFκB signaling [65].

## Effects of AGEs on Resident Skin Cells

AGEs have been shown to affect various functions of skin cells in vitro. They decrease proliferation and enhance apoptosis of human dermal fibroblasts, an effect which is at least partly RAGE dependent and correlates with the activation of NFκB and caspases [85]. In keratinocytes, AGEs decrease cell viability and migration and induce the expression of proinflammatory mediators [86]. Moreover, AGEs are able to induce premature senescence in human dermal fibroblasts and in normal human keratinocytes in vitro [108–110]. Collagen and ECM protein synthesis have been also found to be decreased, while the expression of MMPs is induced [65]. Dicarbonyls such as glyoxal and methylglyoxal impair the signaling of epidermal growth factor receptor (EGFR), a receptor controlling various cellular functions such as proliferation, differentiation, motility and survival, by formation of EGFR crosslinks, blocking of phosphorylation and impaired activation of ERKs and phospholipase C [111]. Various other growth factors or proteins significant for cellular functions, like bFGF, may be glycated inhibiting their functions [105]. In the context of extrinsic aging, AGEs seem to render cells more sensitive to external stimuli, as UVA irradiated fibroblasts and keratinocytes exhibit decreased viability after exposure to AGEs [112].

## The Role of Oxidative Stress

Oxidative stress has been widely accepted to mediate the deleterious effects of solar radiation in the skin during photoaging. Interestingly, in vitro exposure of AGEs to UVA irradiation leads to formation of ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radicals [84]. AGEs can lead to ROS formation in cells by various ways. They can stimulate NOX to induce production of superoxide anion or they can compromise cellular antioxidant defense systems, e.g. inactivation of Cu-Zn-SOD by cross-linking and site-specific fragmentation of this molecule [107]. Moreover, AGEs are themselves very reactive molecules. As early as during their crosslinking reactions they can act as electron donors leading to formation of superoxide anions [113]. Glycation of proteins creates active enzyme-like centers (cation-radical sites of crosslinked proteins) able to catalyze one-electron oxidation-reduction reactions leading to ROS generation with or without presence of oxygen or transition metals such as iron and copper [113–115]. Finally, autofluorescent AGEs, such as pentosidine, can act as endogenous photosensitizers leading

to increased ROS formation after UVA irradiation of human skin. UV irradiation of human keratinocytes and fibroblasts in the presence of AGEs led to increased ROS formation and decreased proliferation in vitro [112].

## Skin AGEs as Biomarkers of Aging

As AGEs have been etiologically implicated in aging and aging-related pathologies, the idea of using them as biomarkers is appealing. AGEs in the skin have been initially measured by western blots (WB) with polyclonal antibodies or by autofluorescence measurements of skin biopsies, thus restricting the wide use of these measurements. An AGE-Reader (DiagnOptics B.V., Groningen, The Netherlands) has been introduced some years ago as a new, non-invasive method to measure in vivo the skin content of AGEs based on their characteristic autofluorescence [116–118]. Until now it has been shown that skin autofluorescence positively correlates with various diabetes-and age-related complications such as micro and macrovascular complications, renal disease, cardiovascular events, overall mortality, age-related macular degeneration and chronic renal disease [117, 119, 120]. Skin glycation has been proposed as a prognostic factor for the development of diabetic complications [121]. Lately it was shown that skin autofluorescence increases with chronological aging and correlates with skin deposition of AGEs, making this method a potential tool in investigating the effect of various anti-aging products of the cosmetic industry [122].

## Dietary Advanced Glycation End-Products (d-AGEs)

A large database of different food items and their AGE contents has been created by measuring CML with ELISA [18, 100]. In general the reported CML contents are correlated with corresponding levels of methyl glyoxal (MG)-derivatives [18]. AGE content of foods as determined by CML and MG levels shows a highly significant linear correlation ($r = 0.8$, $P = 0.0001$) prepared by different cooking techniques. The highly significant internal correlation between two chemically distinct AGEs (CML and MG) in a variety of foods prepared by different methods validates the methodology applied and supports the choice of CML levels as a useful marker of d-AGE content.

As with CML, foods high in protein and fat contained higher amounts of MG than did carbohydrate-rich foods. Recent studies indicate that the meat group contains the highest levels of AGEs because meats are served in larger portions as compared to fats which tend to contain more dAGE per gram of weight. When items in the meat category prepared by similar methods were compared, the highest dAGE levels were observed in beef and cheeses followed by poultry, pork, fish, and eggs. Lamb ranked relatively low in dAGEs compared to other meats.

Higher-fat and aged cheeses, such as full-fat American and Parmesan, contained more dAGEs than lower-fat cheeses, such as reduced-fat mozzarella, 2% milk

cheddar, and cottage cheese. Whereas cooking is known to drive the generation of new AGEs in foods, it is interesting to note that even uncooked, animal-derived foods such as cheeses can contain large amounts of dAGEs. This is likely due to pasteurization and/or holding times at ambient room temperatures (e.g., as in curing or aging processes). Glycation-oxidation reactions, although at a slower rate, continue to occur over time even at cool temperatures, resulting in large accumulation of dAGEs in the long term. High-fat spreads, including butter, cream cheese, margarine, and mayonnaise, was also among the foods highest in dAGEs, followed by oils and nuts. As with certain cheeses, butter and different types of oils are AGE-rich, even in their uncooked forms. This may be due to various extraction and purification procedures involving heat, in combination with air and dry conditions, however mild they are. The type of cooking fat used for cooking led to the production of different amounts of dAGEs [16].

In comparison to the meat and fat groups, the carbohydrate group generally contained lower amounts of AGEs due to the higher water content or higher level of antioxidants and vitamins in these foods, which may diminish new AGE formation. The highest dAGE level per gram of food in this category was found in dry-heat processed foods such as crackers, chips, and cookies. This is likely due to the addition of ingredients such as butter, oil, cheese, eggs, and nuts, which during dry-heat processing substantially accelerate dAGE generation. Although AGEs in these snack types of food remain far below those present in meats, they may represent an important health hazard for people who consume multiple snacks during the day or as fast meals [123].

Grains, legumes, breads, vegetables, fruits, and milk were among the lowest items in dAGE, unless prepared with added fats. For instance, biscuits had more than 10 times the amount of dAGEs found in low-fat breads, rolls, or bagels [18].

Nonfat milk had significantly lower dAGEs than whole milk. Whereas heating increased the dAGE content of milk, the values were modest and remained low relative to those of cheeses. Likewise, milk-related products with a high moisture index such as yogurt, pudding, and ice cream were also relatively low in AGEs [18].

## Factors Affecting the Rate of Dietary AGEs (d-AGEs) Formation During Cooking

The rate of formation and the diversity of the generated AGEs in food depend on factors such as composition, availability of precursors, presence of transition metals, and availability of pro and antioxidants. Reaction time, processing temperature, concentrations of reactants, availability of water, and pH are particularly well known to have a decisive effect on the rate of the Maillard reaction [124]. As a rule of thumb, the rate of the Maillard reaction at least doubles when the temperature is increased by 10°C. If browning is used to measure the progress of the Maillard reaction, then 4 weeks at 20°C, 3 h at 100°C, and 5 min at 150°C give approximately the same result [125]. Factors like pH [126, 127] and water activity greatly affect the rate of formation of Maillard reaction products (MRPs).

The rate of the Maillard reaction is considered to be low at acidic pH, but increases with increasing pH until a maximum is reached around pH 10 [21]. At higher moisture levels, a decrease in reaction rate is observed due to dilution of the reactants in the aqueous phase. Water is a product of the reaction and it is probable that the law of mass action also leads to a decreased rate of reaction at high moisture levels [128]. Dry heat cooking has been found to promote formation of dietary AGEs as determined by immunological methods. However, AGE formation seems to be reduced by heating in an oven at high humidity, shorter cooking times, lower cooking temperatures, or by the use of acidic ingredients, such as lemon juice or vinegar [16].

## Absorption and Bioavailability

Early animal studies reported that MRPs are at least partially absorbed, and those low molecular weights (LMW) MRPs are absorbed to a higher degree than high molecular weight (HMW) MRPs [129]. The absorption of AGEs into the circulation in humans measured by a nonspecific ELISA method was estimated to be about 10% of ingested AGEs [130]. HMWAGEs need to be degraded by gut proteases before the LMW products are liberated. The bioavailability of the partially degraded HMW AGEs will depend on the size of the associated peptide, type of diet, gut environment, and duration of their presence in the gut. Heat-induced changes in proteins can decrease their susceptibility to degradation by gastrointestinal enzymes, and protein and mineral bioavailability have been shown to be influenced negatively by a heat-treated diet [131–133]. Oral bioavailability is thought to be low (10%), secondary to poor absorption from the gastrointestinal tract, as AGE cross link formation is resistant to enzymatic or chemical hydrolysis [130]. The water solubility and amphoteric properties makes LMWAGEs to be absorbed to extracellular and intracellular compartments than HMWAGEs.

The in vivo distribution of CML and CEL after an intravenous injection in rats showed a temporary accumulation in the liver [134], indicating that they may have high affinity to some specific hepatic proteins. In the study of $^{14}C$ labeled AGEs, it was observed that 60% of the absorbed AGEs were bound in liver and kidney after 72 h, but radioactivity was also observed in lung, heart, and spleen indicating more global distribution and tissue binding [135]. Several animal studies have shown a correspondence between dietary AGE content and serum and tissue AGE levels [136, 137].

Any deterioration in renal function results in AGE accumulation which can lead to endothelial perturbation and hence vascular disease [138]. In vitro studies have proposed that insulin also contributes to AGE elimination from the plasma via the IRS and phosphatidyl-inositol-3-OH kinase (PI3 kinase) pathway [139]. This pathway is thought to be vasculo-protective, leading to a rise in nitric oxide as well as facilitating insulin-mediated glucose transport in adipocytes and skeletal muscle. Recent human studies revealed that about 10% of diet-derived AGEs were absorbed, twothirds of which remained in the body and only one-third of the absorbed AGEs was excreted into the urine within 3 days from ingestion [16, 130, 135].

## Dietary Advanced Glycation End-Products (d-AGEs) and Their Health Implications

Nutrient composition, temperature and method of cooking can affect the formation of AGEs in foods. Fats or meat-derived products processed by high heat such as broiling and oven frying contain more AGEs than carbohydrates boiled for longer periods [100, 140]. That is, in the absence of lipids and proteins or heat, sugar content does not necessarily correlate with AGE values in the food. And, the absence of sugars does not necessarily predict low AGE content, as in preparations containing preformed AGElike caramel additives [130].

Food-derived AGEs induce protein cross-linking and intracellular oxidant stress similar to their endogenous counterparts when tested in vitro using human-derived endothelial cells [141]. These pro oxidant and pro inflammatory properties are also found in the circulating AGE fractions derived from these exogenous AGEs. Experiments performed in different animal models have established a significant role for dietary AGEs in inducing T1DM in non-obese diabetic (NOD) [136]. In a group of diabetic subjects, dietary AGE restriction was associated with significant reduction of two markers of inflammation, plasma C reactive protein (CRP) and peripheral mononuclear cell TNF-α, as well as of VCAM-1, a marker of endothelial dysfunction [92]. These observations were later extended to chronic renal failure patients on maintenance peritoneal dialysis, in whom dietary AGE restriction was associated with a parallel reduction of serum AGEs and CRP [142]. The parallel changes of serum AGEs and CRP following dietary AGE modifications are highly suggestive of a role for dietary AGEs in inducing inflammation [16].

## Role of Food-Derived AGEs in Vascular Complications in Diabetic Animals

With regards to complications of Diabetes, several different animal models have been used to examine the role of dietary AGEs in the development of kidney disease. In diabetic mouse models, there has been reports of both protective [143] and disparate effects [144] of diets low in AGEs in development of diabetic nephropathy. In remnant kidney models in rats, proteinuria increased during feeding with high AGE diets [145, 146]. Furthermore, high AGE diets were shown to accelerate progression of renal fibrosis [145]. In addition, in a mouse model of obesity, renal impairment developed when high AGEs and a high fat diet were combined. An AGE-poor diet that contained four- to five-fold lower AGE contents for 2 months also decreased serum levels of AGEs and markedly reduced tissue AGEs and RAGE expression, numbers of inflammatory cells, tissue factor, VCAM-1, and MCP-1 levels in diabetic apolipoprotein Edeficient mice [147].

## Role of Food-Derived AGEs in Ageing

Aging is associated with increased oxidative stress generation and AGE formation [71]. A life-long restriction of AGE containing diet reduces oxidative stress generation and AGE accumulation which are associated with RAGE and p66 suppression, resulting in extension of lifespan in mice [71]. Oral intake of AGE-containing foods also determines the effects of calorie restriction on oxidant stress, age-related diseases, and lifespan [148]. These observations suggest that restriction of AGE-rich diet may be a novel therapeutic target for prevention of age associated various disorders.

In food analyses, CML has been the most widely used marker for AGEs [149]. The CML content of the same food item can be increased up to 200-fold by increasing the temperature and conditions used in cooking. The CML concentrations of various foods vary widely from about 0.35–0.37 mg CML/kg food for pasteurized skimmed milk and butter to about 11 mg CML/kg food for fried minced beef and 37 mg CML/kg food for white bread crust. Fried meat, sausage, and cookies are high in CML [150]. Other foods that are high in AGEs include many commercial breakfast cereals [151], roasted nuts and seeds [152], ice cream [153], and barbecue sauces [154]. High concentrations of MG, an intermediate product of the Maillard reaction, are found in commercial soft drinks that contain high fructose corn syrup [144]. MG is reactive and readily modifies lysine or arginine residues of proteins to form CEL and hydroimidazolones. Pasteurized milk and sterilized milk contain much higher CML concentrations than raw milk [32]. Evaporated whole milk contains high concentrations of CML, probably due to the high temperatures used in processing the milk. Infant formula contains high concentrations of AGEs [155]. Commercial infant formulas contain a 70-fold higher level of CML than human breast milk, and infants fed infant formula had significantly high plasma CML than breast-fed infants [156]. Foods that are either eaten raw or cooked at lower temperatures are relatively low in AGEs, and such foods include raw fruits and vegetables, raw fish, raw nuts, yoghurt, tofu, pasta, boiled rice, boiled potatoes, and other boiled or simmered foods.

Other processes, besides the formation of AGEs, also take place in food during cooking. It is well-known and described in the literature that heating of food induces degradation and oxidation of heat-sensitive compounds, including vitamins and other bioactive compounds [157–159]. A high versus low AGE diet made by differences in heat treatment will, therefore, have dissimilar content of such compounds and this has also been confirmed when it has been measured in intervention studies [160]. This is a problem, because effects of high AGE diets cannot be directly related only to the AGE content. It cannot be ruled out that a lower content of a range of heat-sensitive nutrients in the diet, e.g., vitamin C, E, and thiamine, could also contribute to these negative effects. Accordingly, AGE levels in body fluids

might be markers of the inflammatory and oxidative burden. For example, marginal thiamine deficiency has been shown to increase both markers of oxidative stress and of reactive dicarbonyls [161], and vitamin B6 can also affect AGE formation. Furthermore, extensive heat processing of food can generate Maillard-derived antinutritional and toxic compounds [162, 163]. Such compounds include acrylamide [164, 165], heterocyclic aromatic amines [166] and 5-hydroxymethylfurfural [167], all of which are suspected carcinogens. Thus, simply referring the effects of a less heat-treated diet to effects of AGEs is problematic; the consequences of cooking for the concentrations of AGEs as well as other heat-derived compounds are not tested in the majority of the dietary AGE studies. Only one study has reported the content of acrylamide and 5 hydroxymethylfurfural and they were found to be significantly higher in the high AGE diet [168].

Nevertheless, this shows there is a large range of potentially harmful compounds generated by heat and points to the essential problem with identifying the active compounds. Harmful effects of high AGE diets cannot be directly related to the AGE content. Studies with well-defined compounds outside a complex food matrix (e.g., synthetically produced AGEs) are needed to identify individual effects. Moreover, AGEs are often investigated and discussed as a whole, even though they are a large and heterogeneous group of compounds. The heterogeneity of AGEs makes it difficult to conclude which of these compounds are biologically active and exert which specific effects in vivo. Within the large range of MRPs, not only AGEs have been identified, but also compounds with potential beneficial effects have been described. Melanoidins have been associated with health benefits in some studies and antioxidative properties of MRPs have been observed in a human intervention study [16, 28].

## Anti-AGE Strategies: Current Knowledge and Future Perspectives

Since the emergence of AGEs as an important pathogenetic factor in Diabetes and aging the development of strategies against AGEs has been in the center of scientific interest. Substances able to prevent or inhibit formation of AGEs, as well as agents able to break already formed AGEs or those antagonizing their signaling have been identified. Some of them are already being tested in clinical trials [169, 170].

### Substances Preventing or Inhibiting AGE Formation

Aminoguanidine was one of the first substances identified limiting the formation of AGEs [171]. Aminoguanidine is a nucleophilic hydrazine and its anti-AGE properties result from trapping of early glycation products such as carbonyl intermediate compounds. It has no effects on more advanced stages of glycation. Despite its potential effects in attenuating various diabetes- and age-related complications in animal models, its use in clinical practice is limited due to adverse effects in clinical

trials with diabetic patients [172]. In an in vitro skin aging model it could attenuate collagen glycation, however its effects against AGE induced collagen modification in vivo have been contradictory [173–175]. Studies on topical application of aminoguanidine in the skin are lacking.

Different AGE inhibitors suppress AGE formation at different stages of glycation. For example, aspirin (acetylsalicylic acid) is known to inhibit glycation by acetylating free amino groups of a protein, thereby blocking the attachment of reducing sugars [176, 177] at the early stage of the glycation process. The inhibitory activities against AGE formation of various vitamin B1 and B6 derivatives such as pyridoxamine [178–180] and thiamine pyrophosphate [181] have mainly been attributed to their abilities to scavenge reactive carbonyl compounds [32, 180].

Pyridoxamine, a naturally occurring vitamin B6 isoform, seems to be another tool in the fight against AGEs. Pyridoxamine traps reactive carbonyl intermediates, scavenges ROS and in addition inhibits post-Amadori stages of AGE formation [182]. It has shown promising results in a phase II clinical trial against diabetic nephropathy [183]. Oral intake of pyridoxamine resulted in potent inhibition of skin collagen CML formation in diabetic rats. In addition, penicillamine could reduce the level of AGEs through decreasing the formation of Amadori products [175, 184, 185]. However, its potential against skin aging remains to be shown.

### "AGE Breakers"

Chemical substances and enzymes able to recognize and break the Maillard reaction crosslinks have been identified. Such chemical AGE breakers are dimethyl-3-phenayl-thiazolium chloride (ALT-711), N-phenacylthiazolium and N-phenacyl-4,5-dimethylthiazolium [183]. They have been developed to chemically break the prototypical Maillard reaction crosslink via a thiazolium structure [183]. Promising results against cardiovascular complications in Diabetes and aging have been reported, although their actual ability to cleave existing protein crosslinks in tissues has been questioned [184–187].

Interference with intrinsic AGE-detoxifying enzymes like FAOXs, FN3K and the enzymatic system of Glo is another interesting strategy to remove AGEs, as enzymes recognize specific substrates and may be associated with fewer side effects [57, 188, 189]. There are a lot of data supporting the significance of these enzyme systems in aging. As noted above decreased Glo I activity and increased accumulation of AGEs with age have been shown in many tissues and animals [56]. Overexpression of Glo I significantly inhibits hyperglycemia-induced intracellular formation of AGEs in bovine aortic endothelial cells and in mouse mesangial cells by reduction of intracellular oxidative stress and apoptosis [190, 191]. A potential in vivo beneficial effect of Glo I against AGEs could be also shown in transgenic rats [192]. Interestingly, it has been recently shown that Glo I is transcriptionally controlled by Nrf2, and that pharmacological Nrf2 activators increase Glo I mRNA and protein levels as well as its activity [56]. The pharmacological induction of such enzymes could represent a novel future strategy against AGEs. Fructosamine phosphokinases

are relatively new enzymes and currently under investigation, and until now no inductors or activators of their expression have been found [35]. FAOXs, on the other hand, are not expressed in mammals, and their potential use in humans by enzymatic engineering remains to be discovered [58].

## Nutriceuticals

Since oxidation steps are crucially involved in formation of many AGEs, substances with antioxidative or metal chelating properties, may also have antiglycating activities [193]. Thus, a lot of interest has been directed to nutrients and vitamins, so called "nutriceuticals," as natural tools against AGEs [170, 194].

Accordingly, an increasing list of natural antioxidants and chelating agents such as ascorbic acid, α-tocopherol, niacinamide, pyridoxal, sodium selenite, selenium yeast, trolox, rivoflavin, zink and manganese has been shown to inhibit glycation of albumin in vitro [195]. Alpha-lipoic acid was able to reverse tail tendon collagen glycation in fructose-fed rats, an effect which was attributed to its endogenous antioxidant action, its ability to recycle ascorbic acid, α-tocopherol and GSH as well as to its positive influence on glucose uptake and glycaemia [196]. Green tea, vitamins C and E and a combination of N-acetylcystein with taurine and oxerutin could inhibit skin collagen glycation in mice [194, 197]. Another compound, the green tea-derived polyphenol and flavonoid epigallocatechin-3-gallate revealed also promising in vitro effects by antagonizing AGE-induced proinflammatory changes [198]. In healthy human subjects, supplementation of vitamin C significantly decreased serum protein glycation [199].

Many spices and herbs were shown to inhibit glycation of albumin in vitro, among them ginger, cinnamon, cloves, marjoram, rosemary and tarragon [200]. Their protective effects correlated with their phenolic content. Recently, in vivo beneficial effects of some of these compounds were shown in zebrafish [201]. Other promising compounds include blueberry extract and naturally occurring flavonoids, such as luteolin, quercetin and rutin, which can inhibit various stages of AGE formation [202, 203]. Blueberry extract, an AGE-inhibitor and C-xyloside, a glycosaminoglycan synthesis stimulator, were tested for 12 weeks in female diabetic subjects. This treatment resulted in significant improvement of skin firmness, wrinkles and hydration although it failed to show a significant decrease in the cutaneous content of AGEs [202].

In one of the few human studies successfully conducted on anti-AGE therapeutics, L-carnitine supplementation for 6 months in hemodialysis patients significantly decreased levels of AGEs in the skin [204]. L-carnitine, which is naturally abundant in meat, poultry, fish, and dairy products, is an antioxidant. Furthermore, it may function synergistically to neutralize oxidative stress when given with α-lipoic acid [205].

As a well-known nutraceutical product, grape seed extract (GSE) is an abundant source of catechins and proanthocyanidins with a strong antioxidant and free radical scavenging activity [206]. Peng et al. [209] studied the effects of GSE on the

formation of Nε—(carboxy-methyl) lysine (CML) in bread. Besides introducing antioxidant activity to bread, GSE also appeared to attenuate CML content in bread crust. In particular, adding 600 and 1000 mg of GSE to bread (500 g) led to over 30% and 50% reduction in bread crust CML content, respectively. Strong antioxidant activities of catechins and proanthocyanidins abundant in GSE may contribute to the reduction of CML in GSE-fortified bread [207]. On the other hand, catechins and proanthocyanidins proved to be able to scavenge the intermediate dicarbonyls (such as MG, glyoxal) [208, 209] in the glycation process, which may also decrease the CML content of GSE-fortified bread.

## Caloric Restriction and Dietary Measures

As nutrition is an important factor in skin aging, dietary caloric restriction may be effective in preventing accumulation of AGEs in the human body. In mice restriction of caloric intake increases lifespan and delays many age-related dysfunctions by altering stress response and influencing the expression of various metabolic and biosynthetic genes [210]. Dietary restriction could significantly decrease the levels of AGEs in rat and mice skin collagen [211, 212]. Skin collagen glycation and glycoxidation inversely correlated with lifespan whereas caloric restriction led to decreased accumulation of AGEs and increased lifespan [213]. Dietary restriction may not be a pragmatic option in humans; however a restriction in intake of dietary "glycotoxins" may be more feasible. As outlined above these dietary glycotoxins derive from nutrition. In humans dietary glycotoxins significantly increase concentrations of systemic inflammatory mediators like TNFα, interleukin (IL)-6 and C-reactive protein and are thus considered as diabetogenic, nephrotoxic and proatherogenic [92, 214]. Dietary intake of AGEs correlates with serum AGEs and can induce systemic oxidative stress, increase RAGE expression, decrease antioxidant levels and shorten lifespan in mice [148]. A diet with a low content in AGEs could reduce circulating AGEs and inflammatory biomarkers in patients with Diabetes and renal failure thus seeming to be an important supportive therapy in Diabetes [215, 216]. In mice low dietary AGEs had beneficial effects in wound healing and other DM-associated pathologies [136]. There are no studies investigating the effects of AGE-poor diets on skin aging in humans. However, it has been shown that skin collagen glycation positively correlates with blood glucose levels in Diabetes and that intensive treatment can reduce the levels of skin glycation, implicating that a diet low in AGEs may have a beneficial effect on skin glycation [217, 218].

## Targeting RAGE

Another potential strategy against excessive accumulation of AGEs could be the antagonism of RAGE [219]. Possible approaches include gene knock-down of RAGE by siRNA or anti-sense and antagonism of RAGE with putative small molecular inhibitors against RAGE-induced signaling [67, 219]. Promising effects in

various systems have been shown in vitro and in vivo with neutralizing anti-RAGE antibodies [60]. Since serum concentrations of sRAGE negatively correlate with AGE-induced pathologies, neutralization of AGEs by these decoy receptors of RAGE may be considered as another anti-AGE strategy. Potential protective effects of sRAGE have been shown in various Diabetes and inflammatory models [60, 62, 63, 220]. Interestingly, sRAGE could also attenuate impaired wound healing in diabetic mice. Therefore, studies will be needed to investigate an analogous effect on skin aging [221].

### Others

Molecular chaperones like carnosine have lately shown promise in improving skin appearance in various studies at least in part by reducing the amounts of skin AGEs [222–224].

## Combating AGE with Diet

Nearly 70 years ago, Urbach and Lentz reported that the level of sugar both in the blood and in the skin is decreased with a diet low in sugar [225]. Although its significance was not appreciated at the time, this finding demonstrated a quintessential connection between diet and skin health. We now understand that food is a source of both monosaccharides that, in high amounts, catalyze the production of AGEs in the body, and preformed AGEs [226].

Preformed AGEs are absorbed by the gut with approximately 10−0% efficiency. They can then enter the circulation, where they may induce protein cross-linking, inflammation, and intracellular oxidative stress. The end result is the amplification of a similar *"vicious cycle",* which may be as detrimental as the consumption of excess dietary sugar [227]. Interestingly, preformed AGEs largely result from exogenous synthesis mediated by the food cooking process. Grilling, frying, deep fat frying, and roasting methods are all known to produce higher levels of AGEs in food. In contrast, methods of preparation that are water-based, such as boiling and steaming, produce a logarithmically lower amount of AGEs [21].

A diet low in AGEs correlated with a reduction in inflammatory biomarkers (i.e. TNFα, IL-6 and CRP) in diabetic human patients, as well as an improvement in wound healing and other diabetes-associated sequelae in mice [136, 216]. Other authors have cited the relatively youthful appearance that is often associated with the elderly Asian population as evidence of the long-term impact of employing water-based cooking practices, which are characteristic of Asian cooking [226].

The varying conditions of water and heat play a significant role in the production of dAGE content. As scrambled eggs prepared in an open pan over medium-low heat had about one half the dAGEs of eggs prepared in the same way but over high heat. Similarly, poached or steamed chicken had less than one fourth the dAGEs of roasted or broiled chicken. Moreover, microwaving also did not raise dAGE content

to the same extent as other dry heat cooking methods for the relatively short cooking times (6 min or less) that were tested. In nut shell, higher temperature and lower moisture levels coincided with higher dAGE levels [18].

Tight glycemic control over a 4-month period can result in a reduction of glycated collagen formation by 25% [226, 227]. Consumption of a low-sugar diet prepared through waterbased cooking methods would limit both the consumption of preformed exogenous AGES and endogenous production through physiological glycation. Avoiding foods that result in higher levels of AGEs, such as donuts, barbecued meats, and dark-colored soft drinks, can be an effective strategy for slowing "*sugar sag"* [21].

Beans are recommended as suitable foods for diabetic patients in the past mainly for their high fibre and protein contents. Four kinds of beans including mung bean (Vigna radiata) black bean (Phaseolus vulgaris L.), soybean (Glycine max) and cowpea (Vigna unguiculata) were investigated for trapping of MG, a key intermediate compound for the formation of AGEs. The aqueous alcohol extracts of all beans examined have showed significant inhibitory activities at a concentration of 500 ppm with 80.4% inhibition for mung bean, 72.1% for black bean, 70.1% for soybean, and 67.3% for cowpea extract, respectively [208]. Various phenolic antioxidants from plant extracts have been found to inhibit the formation of AGEs, and their inhibition of free radical generation in the glycation process and subsequent inhibition of modification of proteins have been considered as the major mechanisms for mediating their anti-glycation activities. Total phenolics were determined and it was found that mung bean extract had the highest phenolic content and anti-glycation activities of these beans were highly correlated with their total phenolic contents ($R2 = 0.95$). Two major phenolic compounds from mung bean, vitexin and isovitexin were studied for their activities in direct reapping of MG [208].

Low or acidic pH also arrests the new AGE development. For example, beef that was marinated for 1 h in lemon juice or vinegar formed less than half the amount of AGEs during cooking than the untreated samples [18] Green tea is known well for diabetic people in several ways. It reduces blood glucose level; improves sensitivity to insulin and enhances antioxidant defenses [228, 229]. Furthermore, green tea inhibits the formation of AGEs in an in vitro bovine serum albumin (BSA)/glucose system and in the collagen of aged rats and diabetic rats.

Of interest, several culinary herbs and spices are believed to be capable of inhibiting the endogenous production of AGEs (specifically fructose-induced glycation). These include cinnamon, cloves, oregano, and allspice [195, 196, 200]. Other dietary compounds that have been linked to inhibition of AGE formation based on in vitro data and preliminary animal models include ginger, garlic, α-lipoic acid, carnitine, taurine, carnosine, flavonoids (e.g., green tea catechins), benfotiamine, α-tocopherol,niacinamide, pyridoxal, sodium selenite, selenium yeast, riboflavin, zinc, and manganese [195, 196, 200]. The cosmeceutical industry has taken notice of this data, and several have recently released topical products containing carnosine and α-lipoic acid, with claims related to anti-AGE formation [227]. However, data is lacking as to whether topical administration of these compounds is as effective as dietary delivery in slowing the aging process.

Since glycation is accelerated in the presence of ROS, antioxidants should theoretically be effective in limiting the production of new AGEs. They may also impact AGE-induced tissue damage. One intriguing study looked at the effects of the antioxidant resveratrol. Popularly known for its abundance in red wine, resveratrol is a natural phenol produced by several plants in response to injury and is found in the skin of grapes, blueberries, raspberries, and mulberries. In one study, resveratrol inhibited AGE-induced proliferation and collagen synthesis activity in vascular smooth muscle cells belonging to strokeprone rats [230]. Another study found that it decreased the frequency of DNA breaks in MG treated mouse oocytes. Although resveratrol does not appear to reverse the glycation process itself, these studies suggest that it can reduce AGE-induced tissue damage [231]. While these findings are promising, to our knowledge these laboratory results have not yet been demonstrated in human studies.

Numerous traditional herbal infusions, including Luobuma (Apocynum venetum L.), Nagarmotha (Cyperus rotundus), Mate (Ilex paraguariensis) and Guava (Psidium guajava L.) exhibit potent anti-glycation capacities [232–235]. All herbal infusions inhibited the glucose-mediated formation of fluorescent AGEs in a dose-dependent manner at dilutions of 10-fold to 40-fold. At a ten-fold dilution, balm, mint, black tea, green tea and sage almost completely inhibited the formation of fluorescent AGEs. At a 20-fold dilution, only balm retained its capacity to inhibit totally the formation of fluorescent AGEs. Accordingly, comparing the antiglycation capacities of different herbal infusions based on the experimental results obtained from a 40-fold dilution seems logical. At a 40-fold dilution, the anti-glycation capacity of herbal infusions followed the order, balm (89.8%) >mint (47.8%) >black tea (38.0%) >green tea (35.4%), sage (33.4%) and common verbena (30.4%) >rosemary (18.8%) >lemongrass (3.0%) [236].

Based on the current evidence, individuals with Diabetes and/or kidney disease seem to be the population groups deriving most benefit from an AGE-restricted diet and potentially from inhibition of AGE-formation and its associated actions in the body [16, 194, 228, 237].

## How to Win the Battle Against AGEs/ Fight Against AGEs in Kitchen

As modern diets are largely heat processed, they are more prone to contain high levels of AGEs [16]. On an average, the intake of dAGE in a cohort of healthy adults from the New York city areas was found to be 14,700 ± 680 AGE kU/day [101]. By smart food selection and by changing the way of cooking, the level of AGEs could be lowered in the diet. Overall, moving away from foods high in fat, red meat and processed and fast foods and toward a diet focused more on fruits and vegetables, whole grains and lean meats and fish will not only reduce the AGE intake but help to meet other important nutritional goals as well.

Reducing dAGE may be especially important for people with Diabetes, who generate more endogenous AGEs than those without Diabetes and for those with

renal disease, who have impaired AGE clearance from the body [130]. Recently there has been heightened interest in therapeutic diets that are higher in protein and fat and lower in carbohydrate for weight loss, Diabetes and cardiovascular diseases. This type of dietary pattern may substantially raise dAGE intake and thus contribute to health problems over the long term. A safe and optimal dAGE intake for the purposes of disease prevention has yet to be established.

Some tips to win the battle against AGEs in kitchen are as:

- Use of lower cooking temperatures over high cooking temperatures;
- Steming, stewing and poaching are be the cooking methods than frying, grilling and roasting;
- Be wary of browning;
- Higher temperature and lower moisture levels in food during cooking increase dAGE levels;
- Phenolic antioxidants (e.g., in beans) can inhibit the formation of AGEs;
- Addition of acids (e.g., vinegar, lemon juice) lowers AGE levels;
- Green tea inhibits formation of AGEs;
- Cook fresh foods as possible;
- Eat more often at home [16].

## Conclusion

Current evidence from many different disciplines lends strong support to the idea that AGEs contribute to the multisystem decline that occurs with aging. AGEs contribute to inflammation and tissue damage through AGE-RAGE binding. AGEs cross-link collagen and other proteins and thus increase the stiffness of tissues such as the major arteries, heart, bone, and muscle.

There is clearly an abundance of in vitro data and a handful of in vivo animal findings that support various options for dietary therapy directed against "*sugar sag*." However, studies in humans are limited by logistical, ethical, and inherent study design issues. Nevertheless, the role of diet in skin aging is undeniable. As our understanding of how accumulation of AGEs affects a rapidly growing number of pathologies, it is inevitable that our research methods will evolve to better address the challenges that currently seem so discouraging.

In the meantime, awareness of the critical impact of AGE formation in both diabetics and non-diabetics must be extended to all patients, regardless of their current health status. That task begins with clinicians. Dietary counseling should be incorporated into our regular interactions with patients, alongside essential discussions about UV-protection and avoidance of tobacco. After all, these are the three most important known exogenous aging factors. Their common grouping is reflective of their interconnected nature and their action in concert to disturb homeostasis.

Finally, there is ample evidence that AGEs play an important role in skin aging. There are also numerous studies investigating potential substances against excessive accumulation of AGEs in tissues. Some of these studies have already

shown protective effects against diabetic complications. Modification of intake and circulating levels of AGEs may be a possible strategy to promote health in old age, especially because most Western foods are processed at high temperature and are rich in AGEs. As controlled human studies investigating the effects of these anti-AGE strategies against skin aging are largely missing, this is a hot field for future researches.

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