Review

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Biomarkers in Medicine

Advanced glycation end products as biomarkers in systemic diseases: premises and perspectives of salivary advanced glycation end products

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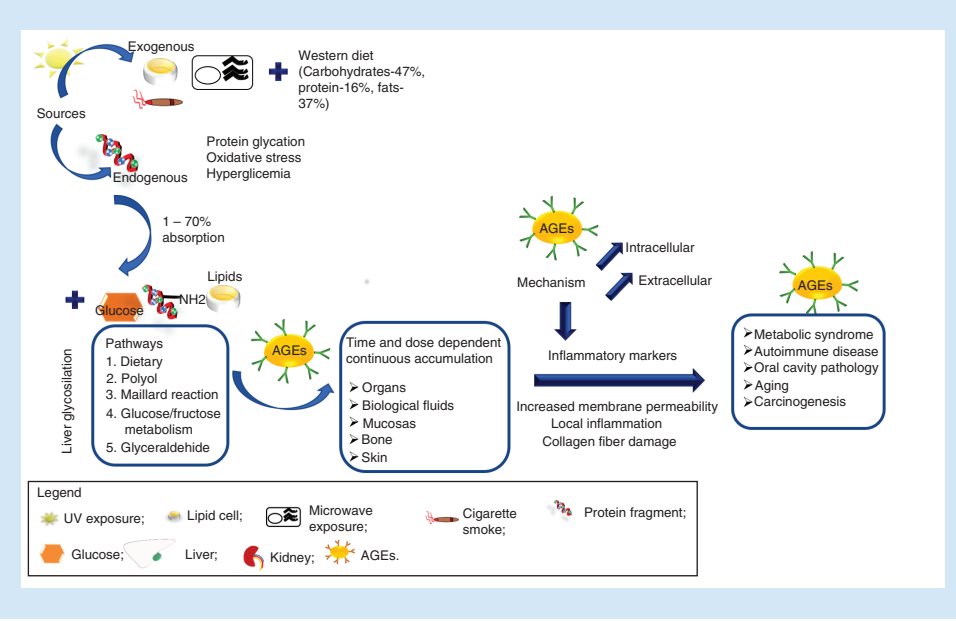
Advanced glycation end products (AGEs) are glycated proteins associated with high dry temperature food processing, coloring and flavor modification of food products. Previous studies on diet-related disease support the role of the glycation products as biomarkers in local and general proinflammatory response. Exogenous and endogenous AGEs are involved in chronic low-level inflammation, which underlies the onset of metabolic syndrome influenced by food intake, there by demonstrating their implication in diet-related pathologies. Although studies have revealed a strong association between the accumulation of AGEs and the occurrence/worsening of metabolic diseases, their routine use for the diagnosis or monitoring of local and general disease has not yet been reported.



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Advanced glycation end products (AGEs) were synthesized for the first time *in vitro* in 1912, by French Louis-Camille Maillard; later, in 1953, JE Hodge divided the reaction, and in 1986, the formation of reducing sugar-derived carbonyl products was added to the Maillard chain reaction [1,2]. AGEs are represented by structures formed via glycation – a nonenzymatic reaction [3] between sugars with a free amino or ketone group and proteins or lipids. This complex and difficult to control reaction is involved in food taste and color modification, especially during processes such as baking, toasting and almost all animal protein cooking processes. In medical research, AGEs have been associated with increased production of reactive oxygen species (ROS), which consequently generate oxidative or carbonyl stress, with negative effects in inflammatory, autoimmune and diet-related diseases (Figure 1).

In the present narrative paper, aspects concerning AGEs' sources, mechanism of action, the implication in systemic and diet-related disease, and the aging process as a subsection will be discussed. A current approach based on salivary AGEs as an inflammatory process biomarker will be presented, as well as anti-AGEs downregulation strategies, tested either *in vitro* or *in vivo*.

AGE production sources, chemistry & metabolism

AGEs sources

AGEs can be classified according to their provenience, fluorescence and cross-linking ability. One of the classifications [4] include fluorescent AGEs, such as GOLD, glyoxal-lysine dimmer; GOLDIC, (2-ammonio-6-([2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1-himidazol-5-ylidene]amino)hexanoate); MOLD, methyglyoxal-lysine dimmer; MODIC, (2-ammonio-6-([2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1-himidazol-5-ylidene]amino)hexanoate); MRX, 8-hydroxy-5-methyldihydrothiazolo(3,2-α)pyridinium-3-carboxylate; pentosidine; cross-line and nonfluorescent noncross-linked AGEs such as carboxymethyl-lysine (CML), carboxyethyllysine, pyrraline and imidazole. AGEs sources can be endogenous and exogenous (Figure 2).

Endogenous AGEs are generated by protein glycation due to oxidative stress and hyperglycemia, which are commonly associated with diabetes mellitus (HbA1c). In hyperglycemia, glucose converts to fructose through an enzymatic process. Under aldosereductase and sorbitol dehydrogenase reaction, fructose accumulates in tissues and decreases glyceraldehyde-3-phosphate (GAP) activity, thus enhancing augmentation of glyoxal (GO) and methylglyoxal (MG) AGEs precursors, GAP and DHAP [5]. It was observed [6] that protein glycation takes place in the human body similarly with processed food, leading to HbA1c. Carbonyls and dicarbonyls groups from thermally prepared food are found in a high percentage in human tissues [7].

The exogenous sources include nutrients – dietary AGEs (dAGEs), ultraviolet exposure, cigarette smoke compounds, microwaves and ultrasounds. dAGEs are easily obtained by ingesting nutrients that are part of the Western diet (carbohydrates – 47%, lipids – 37%, protein compounds – 16%). Nutrients rich in lipids and proteins have higher AGEs concentration compared with carbohydrates [8]. Regarding the temperature and cooking method on dAGEs, dietitians [9] evaluated CML as an AGEs biomarker in 250 sorts of nutrients, using the next preparation methods: boiling (100°C), frying (180°C), broiling (225°C), roasting (177°C) and oven frying (230°C). In the evaluated food, CML levels were the highest in oven frying, followed by deep frying, roasting and water boiling. Besides the cooking conditions, time is also an important factor in the amount of resulting glycation products. Helou *et al.* [10] showed that bread baked at 250°C for 9 min led to 19.6% more CML compared with 200°C for 12 min. Microwave heating significantly increased CML production in saccharide-lysine model systems compared with boiling heating [11]. It was estimated that in European patients, 1-day melanoidins-products intake variates from 10 to 12 g per day from all sources, where coffee and bread are considered to represent half or more [12,13]. Compared with other sources, the exogenous glycation compounds provided by nicotine-/nornicotine-smoking AGEs are formed in a couple of hours [14]. Subsequently, they bind to connective tissue collagen fibers and low-density lipoproteins (LDL) [15].

The glycation products can have a low-molecular weight (LMW) or high-molecular weight (HMW), formed in the advanced stages of the Maillard reaction [16]. A study conducted by Poulsen *et al.* on a group of 36 rats treated with HMW and LMW dAGEs showed that LMW dAGEs were found in a higher concentration in biological fluids and organs [17]. Because protein-bounded dAGEs are evidenced after digestion, HMW and LMW compounds can

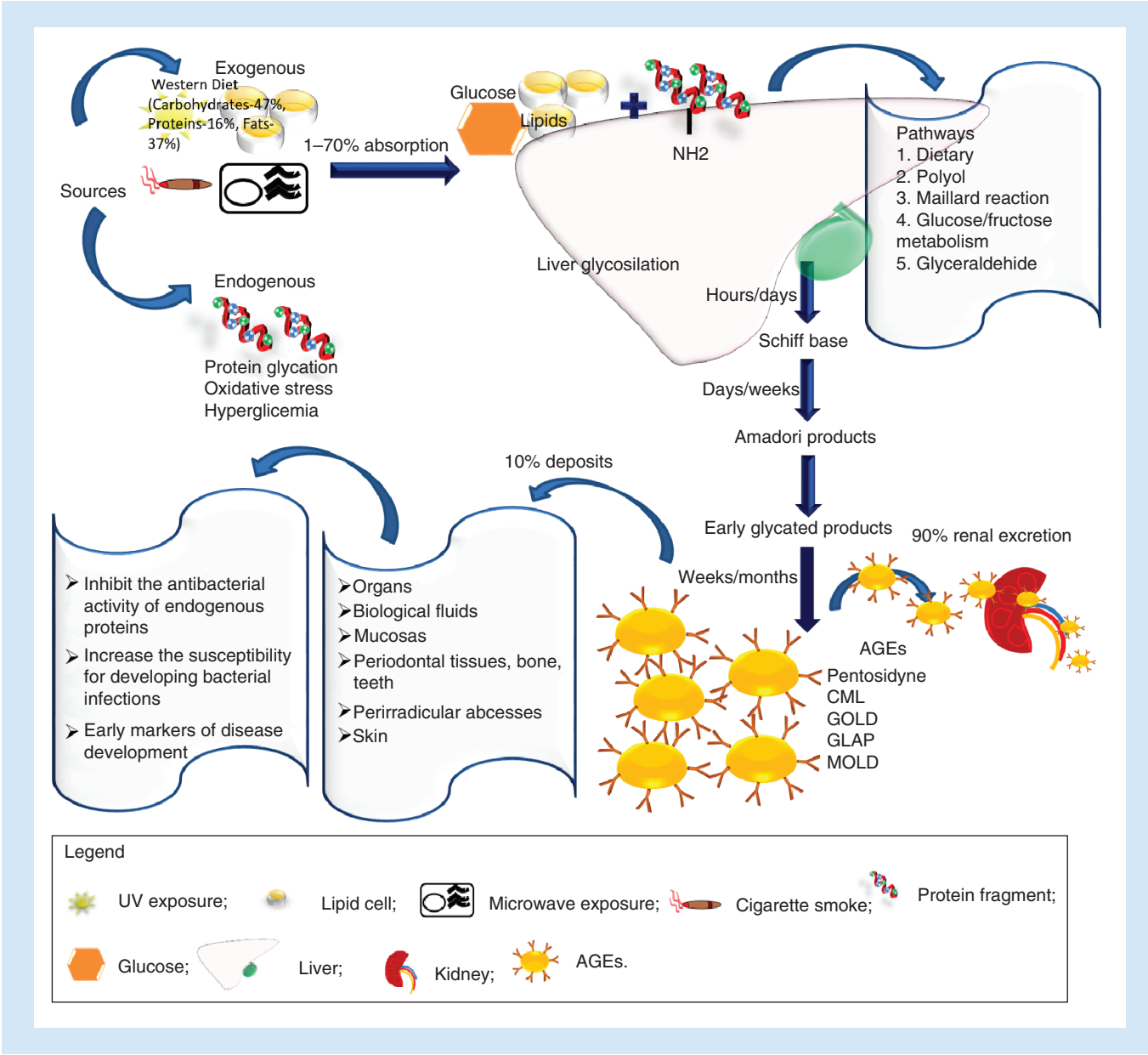

Figure 1. Advanced glycation end product sources, metabolic pathways and effects on storage organs.
AGE: Advanced glycation end products; CML: Carboxy-methyllysine; GLAP: Glyceraldehyde-derived pyridinium compound; GOLD: Glyoxal-lysine dimer; MOLD: Methylglyoxal-lysine dimer.

be evaluated as precursors of melanoidins [18]. Regardless of the source of glycation, the hepatic formation of AGEs occurs in several steps. From the dAGEs ingested, approximately 10% are intravasculary absorbed, from which 30% are urinary excreted. Koschinsky *et al.* performed a 3-day trial to analyze bioavailability of dAGEs in diabetic and healthy subjects, with or without renal dysfunction. Their results showed that approximately 10% of the ingested AGEs were found intravascular [20]. Uribarri *et al.* performed a 3-day dietary restriction on dAGEs intake in a subgroup of five healthy subjects to analyze dAGEs metabolism. The results showed a 30–40% decrease in AGEs serum levels [5]. Other studies [16,19] demonstrated that AGEs bypass absorption due the resistance to hydrolysis of the cross-link bindings in the GI tract.

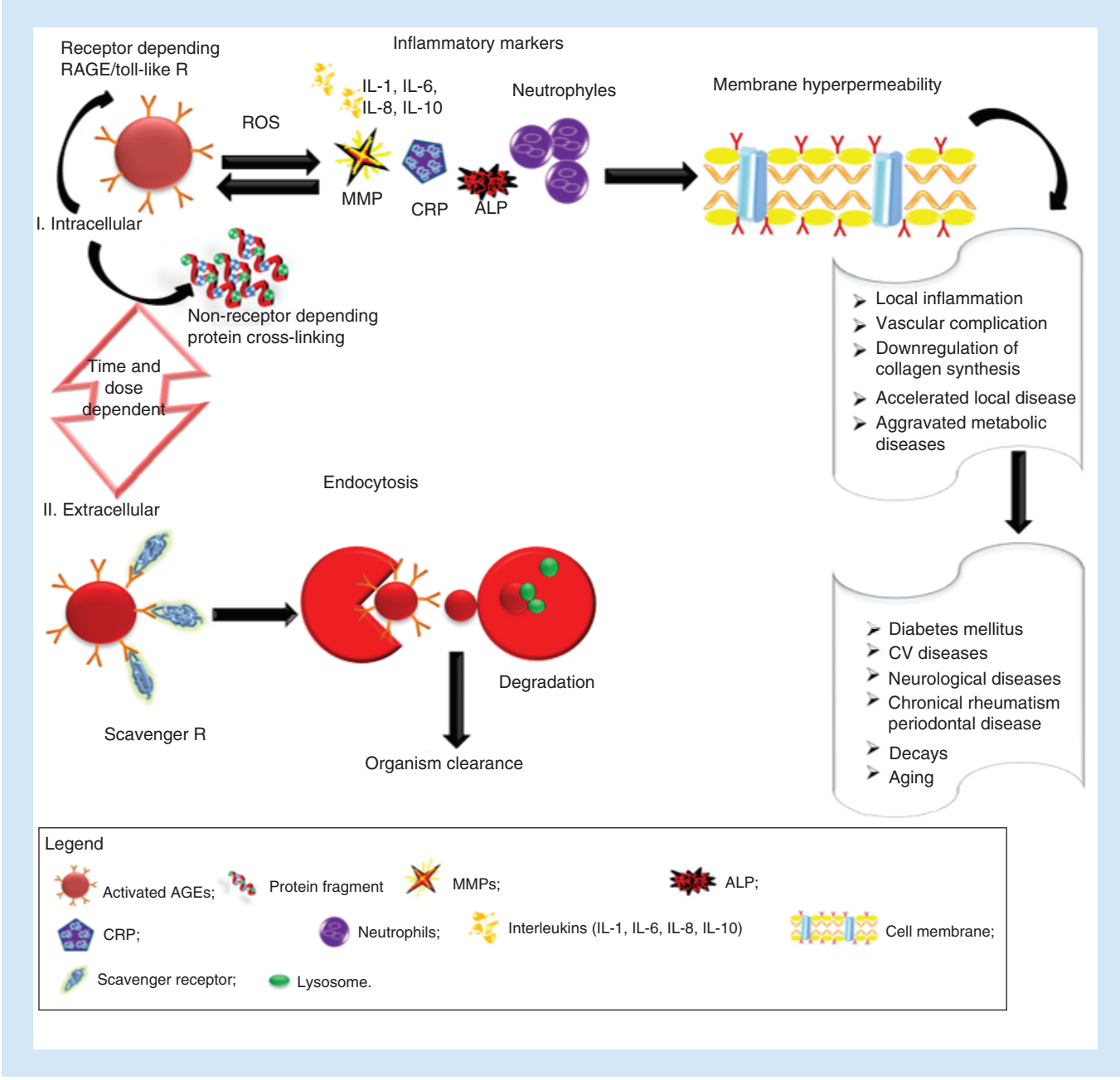

Figure 2. The advanced glycation end products' negative effects on the human body.
AGE: Advanced glycation end products; ALP: Alkaline phosphatase; CRP: C-reactive protein; MMP: Metalloproteinases; ROS: Reactive oxygen species.

DAGEs formation

As mentioned above, Maillard reaction occurs in different cooking methods that are accountable of nutrients color, flavor and attractive appearance. The chemical reaction is between the carbonyl group of a reducing sugar and the amino group of the amino acid. At high temperatures, Amadori compounds are formed, first as stable Maillard reaction products. Of these, furosine is used to measure early glycation, formed after acid hydrolysis of the Amadori products' fructosyl-lysine, tagatosyl-lysine and lactulosyl-lysine produced by the reaction of Lys with lactose, glucose and galactose [21,22]. The final reactions are represented by condensation of sugar and proteins to

polymeric melanoidinic compounds, from which CML is the most representative and a stable product, while GO and MG present reactive activity [23].

The exposure of uncooked food to barbecuing, grilling, roasting, baking, frying, sautéing, broiling, searing and toasting leads to acceleration of dAGEs formation [24]. Moreover, dry heat cooking could cause formation of 10–100% new dAGEs. AGEs content (CML exactly) as kilounits/100 g was analyzed in 549 foods. The study showed that high-heat-treated meat contained the highest AGEs levels, followed by high-fat and conserved cheese [25]. Animal-derived foods also have high AGEs levels, due to the continuous exposure to pasteurization or depositing at room temperature. Carbohydrates, which are rich in water and nonreducing sugars, have a lower expression of AGEs. These statements show that glycation occurs in a high amount in food components. Thoroughly research brought LC–MS as evaluation method for the quantification of dAGEs. There have been validated several databases containing dAGEs ratio (nanogram per gram) [26,27].

DAGEs metabolism

Scheijen *et al.*, in their study, developed a dAGEs database by identification of CML, N-epsilon-(carboxyethyl)lysine (CEL) and 5-(5-hydro-5-methyl-4-imidazolon-2-yl) L-Ornithine d6 (MG-H1) in 190 different nutrients, and then correlated dAGEs with plasma and urinary levels [28]. They found a weak connection between dAGEs and protein-bound plasma AGEs. Birlouez-Aragon *et al.*'s research suggested that when metabolized, dAGEs are divided to nonglycated proteins and free-glycated proteins, from which dAGEs are found circulating in their free form and thus, eliminated through urine [29]. This study is in assent with Li *et al.*'s research [30], who studied the bioavailability of oral gavage pure CML on rat model. The authors found that dietary CML, after entering into circulation, forms CML-bound proteins, which accumulate in organ tissue. Metabolic studies [31,32] showed that plasma AGEs in diabetes and plasma CML levels in renal failure decreased by 30–40% when subjects were on a low glycotoxin diet.

The Dresden Institute for Food Chemistry [33] conducted an experiment in a group of 18 healthy subjects to study the bioavailability and elimination of fructoselysine, pyrroline and pentosidine AGEs. The research team found that free urinary pyrroline and Amadori products had a dietary origin, while free pentosidine, endogenous *in vivo* formation should be taken into consideration. Alamir *et al.* [34] analyzed on a rat model the dietary CML (derived from extruded/nonextruded casein) metabolism. The results showed no differences in plasma CML depending on CML origin; in urine and feces, extruded proteins' levels were lower (38–48%) compared with nonextruded proteins (23–37%). A study that measured furosine accumulation in skin collagen showed that a restrictive diet could lead to limitation of early glycation Amadori products, depending on glycemia [35]. Another study that analyzed age-related skin AGEs for 25 months [36], showed a modification from 18 to 33% in diet-related early glycation AGEs. The accumulation of advanced CML and pentosidine decreased after a few months from the beginning of trial. Although glycation compounds as melanoidins or others rest in the lower GI tract for several hours [37], the low bioavailability of dAGEs is explained by formation of aggregates, cross-link reactions and the lack of specific transport systems [38]. CML and pentosidine accumulate time and dose dependent in human skin and urine and have a higher concentration in diabetic subjects [39]. DAGEs (CML, CEL, MG-H1) resulted from protein and sugar cooked at temperatures higher than 100°C were quantified by LC–MS/MS and tested on human macrophages [40]; the increase in heating time affected macrophages' viability and decreased TNF-α secretion. These trials showed that long-term AGEs deposition depended not only on the circulating sugars but also on ROS levels, lipid oxidation and inflammatory conditions. Chocolate-mixed drink samples containing reducing sugars and proteins showed that the higher the proportion of glucose is, the more CML results [41]. Compared with cold-water-prepared drinks, heating led to 30% more CML.

Endogenous AGEs are formed at a constant body temperature of 36–37°C, they bind to specific ligands and, by chain reactions, they induce an inflammatory state. DAGEs production ranges from low, at approximately 20°C, to high at approximately 230°C. As the temperature and cooking time increase, dAGEs are formed in high amounts and promote strong protein cross-linking. In the human body, glycation products have a large distribution in the soft and hard tissues due to the collagen fibers in the extracellular matrix, and the multiple surface receptors. Both the endogenous and exogenous AGEs are difficult to degrade and metabolize due to the specific chemical linkage reaction, which might explain their continuous and age-related accumulation. Because of their binding to the receptors and the subsequent proinflammatory pathways, AGEs lead to immune cells activation and cellular autophagy, which might maintain and even promote inflammatory cells recruitment, thus resulting in a continuous vicious circle.

Perhaps in the future, people's lifestyle will change, especially related to the eating behavior, taking into consideration the recent findings regarding how dAGEs are formed, the importance of cooking conditions and food storage, and the effects of dAGEs on human body. Public health policies should inform the population, the food companies and the health professionals. People would be advised how to choose between highly processed, tasteful, but unhealthy food and nonprocessed food, with a lower dAGEs content. Further studies will show the population's compliance and the adherence of major food producers to these prevention measures of dAGEs-induced chronic diseases.

AGEs pathogenic mechanisms, ligands & molecular pathways

AGEs can alter tissue integrity by two different pathogenic pathways: receptor-mediated and receptor-independent manner. One receptor for AGEs, known as RAGE, is a multiligand Ig family cell surface molecule, which possesses a 35-kDa polypeptide with a unique sequence at the NH_2-end and HMGB1 (high mobility group box 1) – a chromatin protein, and is the best characterized AGE receptor [42]. RAGE transcription led to 20 splice variants, from which N-truncated (N-RAGE), full-length (FL-RAGE), soluble RAGE (sRAGE) were highly expressed in endothelial cells; sRAGE might as well be produced in FL-RAGE proteolysis condition [43]. RAGE has an extracellular part with 1 V and 2 C-type Ig-like domains, from which the V-domain presents affinity for HMGB-1, AGE, ß-amyloid, S100, and their binding leads to NF-kB, p21ras, MAPK activation, all of them being implicated in cellular programing and oxidative stress generation [44]. V-type domain of the promoter region of RAGE polymorphisms (*T-429C, T-347A*) has been associated with cardiovascular disease risk and *-375T/A* allele showed a protective cardiovascular effect [45]. RAGE interaction with Diaphanous-1 protein activates Gua triphosphatases, which promotes cellular migration [46]; moreover, RAGE interacts with G-protein-coupled receptors, such as formyl peptide receptors, and enhances intracellular AMP consumption, leading to neural cells apoptosis [47].

An important receptor mechanism consists of binding to specific RAGE or immune Toll-like receptors (TLRs), expressed in macrophages, fibroblasts, epithelial and endothelial cells [48]; it was shown that RAGE and TLR4 receptor have a similar receptor-transmission (RT) sequence, both using the same inflammatory cytokine activation pathways. RAGE enhances MAPK pathway that activates NF-kB, induces nitric oxide synthesis, secretion of cyclooxygenase 2, TNF-α and ILs, which are important inflammatory markers [49,50].

sRAGE was reported to have a protective role over RAGE [51,52]. In diabetic patients, metformin administration led to a decrease in serum CML and RAGE, but increased sRAGE [53], and seemed to slow down diabetic atherosclerosis [54]. Several studies focused on the sRAGE, their association with other AGEs and the general pathology, but the exact pathogenic mechanism is not known, and further investigations should be conducted to answer the question.

Other AGE receptors such as AGE-R1, AGE-R2, AGE-R3 and scavenger receptors (which facilitate protein absorption and degradation) [55] may have a role in extracellular AGE clearance by endocytosis and degradation. The nonreceptor mechanism includes cross-linking of protein strands, forming strong covalent bonds, which reduce the elasticity and thus, the functions of proteins [56]. Once formed, they attract and activate inflammatory cells and enzymes; they mediate ROS generation. Increased membrane permeability and chronic inflammation confirm the key role of AGEs as common features linking metabolic abnormalities, inflammatory signaling and cardiovascular dysfunction in cardiometabolic disorders. DAGEs increase tissue hardness and alter serum LDL levels. Their high serum ratio activates cytokines and ROS production, resulting in a strong association with the risk of aggravating existing diseases, and promotes the development of new pathologies.

Perhaps, the classic treatment of chronic diseases will change in the future through the development of new therapeutic agents to block AGEs receptor-mediated pathogenic pathways. Another challenge will be to block AGEs receptor-independent effects. Assessment of RAGE polymorphisms to find the alleles with protective or negative effects on the cardiovascular disease will allow a personalized treatment of the patients, thus avoiding the administration of unnecessary and ineffective drugs.

AGEs & the aging process

AGEs are considered to be a biomarker for aging, due to the continuous accumulation in body fluids and tissues. AGEs are thought to have a role in aging because they accumulate in collagen and elastin fibers in the extracellular matrix, affecting its elasticity. AGEs alter the intracellular function by protein glycation and last, but not least, AGE-RAGE interaction leads to local inflammatory pathways [57]. One of the first experiments that studied these glycation products [58] proposed that the presence of AGEs excites macrophage cytokines, which synthesize TNF-α

and IL-1. Crişan *et al.* studied histological and immunohistochemical aspects of ultraviolet-derived CML in sun-exposed and unexposed skin, in a group of 32 healthy patients subdivided into age categories [59]. Their findings indicated that AGE-CML had a high expression in both exposed and unexposed skin, while CML was 10% higher in exposed skin compared with unexposed skin, proportionally to age. Superficial dermal layers had higher levels of glycation products compared with the deeper layers. Starting from the hypothesis that obese people store more AGEs in tissues and fluids, skin AGE autofluorescence was assessed in Western European patients diagnosed with obesity, smokers or nonsmokers, with or without other metabolic pathologies [60]. The researchers found a difference of almost 10% between obese and nonobese persons, which might be due to variations in the subjects' diet. MG was shown to affect collagen's extracellular matrix during aging, induces endoplasmic reticulum stress and apoptosis by activating the PERK-eIF2α and caspase-12 pathways, and by generating hydrogen peroxide and oxygen-derived free radicals [61]. A systematic review on AGEs influence in wound healing evidenced that AGEs reduce the elasticity and scar thickness, by disarranging collagen structure, producing short and thin fibers [62]. An *in vitro* multiscale mechanical testing using ribose and harvested human tendons' samples showed that AGEs limit fiber–fiber and fibril–fibril sliding and by that the tissue viscoelasticity is strongly diminished [63]. A recent cross-sectional controlled study investigated the association between joint stiffness and AGEs in patients with Type 1 diabetes. Anamnesis, clinical examination and skin biopsies using LC–MS were performed. The results associated joint stiffness with long-term glycosylated hemoglobin and the AGEs MG and pentosidine [64]. Another study analyzed in rat tail and Achilles tendons fibrils mechanics and the effect of MG treatment [65]. Tendons from rats with the age of 4 and 16 weeks were used. The results showed no influence of the age on mechanical properties, but methylglyoxal generated stiffness without fragility of the tested tissues. Regarding hard structures, 170 human bone samples were investigated to evaluate the relationship between pentosidine and total AGEs in cortical and cancellous bone [66]. The result showed a higher pentosidine and total AGEs accumulation in cancellous bone compared with cortical bone. Thomas *et al.* evaluated CML in type I collagen bone samples, using MS, and a protocol including demineralization, heating and trypsin digestion for the assessment of CML in bone tissue [67]. The study showed that CML is from 40- to 100-times higher in bone compared with pentosidine, which is correlated to the skeletal risk of fracture. Serum levels of AGE and sRAGE were investigated in heart failure and dilated ascending aorta diseases. In heart failure patients, serum pentosidine level was shown to be an independent prognosis factor for nonischemic cardiac events; by its interaction with RAGE, pentosidine activates the NADPH oxidase and ROS generation that triggers the NF-kB pathway. This pathway represents a stimulus for the expression of proinflammatory cytokines that are known to be associated to heart failure [68]. In patients with ascending aorta dilatation disease, TGF-ß levels were higher in the tricuspid valve, but no differences in sRAGE serum levels or in valve morphology were found [69], maybe due to the fact that sRAGE have no specificity regarding the topography of the pathology. The above accumulating evidence shows that skin autofluorescence could be correlated with the presence and severity of vascular complications of diabetes and could predict future cardiovascular events and death in patients with diabetes.

AGEs & metabolic disorders

Mastrocola *et al.* gavaged mice with glucose 15% or fructose 15% during 30 weeks, with the purpose of assessing effects on weight, glucose tolerance and lipid profile [70]. The mice became overweight in a proportion of up to 31%, glucose curves moved from the control at every glycemic bump, and the lipid profile was altered, with hepatic steatosis. Glyceraldehyde-derived pyridinium (GLAP) and MOLD were overexpressed in the hepatic tissue of glucose-fed mice, while GOLD and CML were overexpressed in the hepatic tissue of fructose-fed mice. Moreover, the authors suggested the role of CML accumulation in hepatic steatosis, and their hypothesis is supported by other studies using *in vitro* and *in vivo* models [71,72]. In rat hepatocytes culture, the administration of ethanol led to severe downregulation and fatty accumulation in hepatocytes, because of oxidative stress induced by AA-AGEs [acetaldehyde (the main ethanol metabolite)-derived AGEs] [73]. AA-AGEs also led to apoptosis of neural cells, in a dose-dependent manner [74,75].

Worldwide, more than 10% of the population suffer from obesity, and it is estimated that by 2030, this proportion will double [76,77]. The link between AGEs accumulation and obesity development has been recently demonstrated in mice with genetically induced deletion of leptin receptors, which were prone to consume excessive calories and develop obesity and insulin resistance. Obese mice showed high levels of CML trapped by the adipose tissue, while the deletion of RAGE reverted CML accumulation in adipose tissue, increasing the plasma levels; these findings indicated a RAGE-dependent mechanism underlying endogenous AGE-induced obesity [78]. A hypercaloric diet

not only induces obesity but also influences the cognitive function and memory [79,80]. A study conducted by Dahl *et al.* on showed that midlife patients with increased BMI had a decline in verbal and spatial skills [81].

AGEs & neurodegenerative diseases

Several studies demonstrated the connection between dAGEs and neurodegenerative Alzheimer, Parkinson's disease and multiple sclerosis. Percentage differences of AGEs in the cerebrospinal fluid in Alzheimer's disease have been demonstrated [82]. D-galactose administration leads to cognitive disorders in a rodent model [83]. An experiment focusing on the long-term effect of fructose consumption showed that fructose (ten-times more damaging than sucrose) caused high-serum AGEs levels and collagen alteration [84]. The authors suggested that AGEs affected the LDL entry into the brain mediated by astrocytes, and the consequent impairment of neural cell function [85]. Lys represents a high percentage of apolipoproteins, a group of structural proteins in LDL, shown to be highly glycated. As a consequence, they become dysfunctional [86]. In inflammatory stress, the CNS reacts by gliosis, a nonspecific hypertrophy of astrocytes, oligodendrocytes and microglia, viewed as protective feedback. Mastrocola *et al.* evaluated the production of AGEs (marked as CML) in the hippocampal pyramidal cells of mice with a high-fructose diet [87]. The results expressed an increased amount of CML in pyramidal neurons, which activated the RAGE/NF-kB proinflammatory pathway, leading to general reactive gliosis. Considering that AGEs are mostly sugar-reducing sourced, neurological disorders could be associated with increased sweets input – nondiabetic hyperglycemia and instead of serving as a neural energy substrate, sugars become dAGEs and impair the performance of the neurons, with negative effects on cognitive functions. Other substances associated with AGEs, such as ROS and iNOS, could affect cellular energy production and cellular functionality, resulting in memory impairment and aging, either physiological or related to the neurological condition.

AGEs in carcinogenesis

AGEs react with the -NH_2 group of nucleic proteins and could increase the risk of cancer. *In vitro* studies [88] showed that DNA incubation with glucose or a hyperglycemic medium induces mutagenic changes. CML interaction with the markers of carbonyl stress and oxidative stress (ROS and nitrogen species) inhibits growth factors expression in injured tissues, and provides an exposed damaged foundation subject to carcinogenic factors [89]. Hyperglycemia increases RAGE and NADP+ oxidases (NOXs) expression, and this RAGE–NOXs pathway promotes oxidative stress, local hypoxia and VEGF activation, which might stimulate tumoral angiogenesis [90]. In addition to cigarette smoke, RAGE polymorphism is strongly involved in the development of oral squamous cell carcinoma (OSCC), lung and breast carcinoma [91,92]. In gingival carcinoma cells exposed to tobacco smoke, ILs and surface RAGE were found in high amounts after 6 h of exposure [93]. High expression of RAGE in OSCC cell lines was associated with an increase in the depth and local recurrence of oral carcinomas [94,95]. CC genotype *rs2070600 (Gly82Ser) RAGE SNP* was associated to high-serum sRAGE, which might expose RAGE to enzymes and degradation proteins [96]. sRAGE SNP could influence the risk of pancreatic tumoral pathology, where the minor allele of *rs1035786 of RAGE* were associated with reduced pancreatic cancer risk and soluble RAGE concentrations were independently inversely correlated with the 82Ser allele of *rs2070600 of RAGE* and positively correlated with serum CML [97]. In mice model-induced hepatocellular carcinoma, oval cells (OC) liver progenitor cells observed in chronic liver damage, were overexpressed due to HMGB1–RAGE interaction and presented high RAGE levels, while HMGB1 blockade caused a decrease in OC as well as RAGE expression [98].

In tumoral pathogenesis, injured cells release damage-associated proteins, from which HMGB1 is one of the most representative – it is discharged during cell apoptosis or under cytokines, RAGE and Tyrosine kinase receptors (TKRs) stimulation [99]. HMGB1 can be synthesized intracellularly (as a physiologically stimulating growth factor), or extracellularly (with a proinflammatory effect). The association between HMGB1, released by RAGE, and NF-kB p65 increases melanoma inhibitory activity in OSCC [100]. HMGB1–RAGE binding also represents an independent factor in tumoral proliferation, invasion and metastatic potential [101]. RAGE activation by HMGB1 leads to the activation of type 2 macrophages tumoral activity, NF-k-light-chain promotor of B-cells, having as a result cellular growth and malignant features [102]. Moreover, S100A7 and S100A14, calcium-binding proteins, connect with RAGE receptors and promote epithelial–mesenchymal cell migration, cancer cell invasion and metastasis in osteosarcomas and cervical cancer [103,104]; in glioma-conditioned medium, mesenchymal stem cells were stimulated toward a tumoral development S100B-RAGE mediated [105].

AGEs in the saliva & their suitability as biomarker

For the noninvasive diagnosis and monitoring of local and general diseases, saliva, as a biological fluid, is very accessible and easy to collect. Unlike blood collection, which is almost always uncomfortable and painful for the patient, or feces or urine analysis, which makes people feel embarrassed, saliva collection is highly accessible, by a simple spit into sterile recipients. Repeated samples can be collected, and diagnosis and monitoring using this method is an innovative and attractive approach.

Among saliva components, mucins are heavily glycosylated proteins, which are physiologically secreted by epithelial cells in the mouth. Their carbohydrate composition varies from tissue to tissue and is altered in response to mucosal infection and inflammation. Mucins in the oral cavity (including MUC5B, MUC7, MUC19, MUC1 and MUC4) protect the mucosa from pathogenic bacteria and noxious substances but provide favorable conditions to beneficial oral microorganisms through their carbohydrate receptors [106,107]. Salivary mucins are carriers for glycoproteins that have antibacterial activity; they transport these proteins, enable their retention on the surface of the teeth and prevent their degradation by forming complexes. The glycosylation of these proteins is also altered in response to environmental stress.

A study on healthy volunteers assessed the variation of oxidative parameters in unstimulated saliva after mechanical removal of dental plaque, 30 days in a row. Salivary AGEs and advanced oxidation protein products were higher in men than in women, and there was a 60% variation between individuals [108]. Salivary thiobarbituric acid reactive species (TBARS), markers of cell membrane peroxidation, and advanced oxidation protein products were analyzed mostly for their involvement in periodontal disease, as biomarkers of aggressiveness or efficacy of periodontal therapy [109-112]. Gingival attachment is an easily oxidized soft tissue, and markers accumulate in the adjacent saliva. The Comenius Research Unit in Bratislava [113] evaluated in a cross-sectional study of 82 pediatric patients' salivary AGEs using unstimulated saliva, by spectrophotometric and spectrofluorometric measurements. The authors found differences in expression related to gender and oral hygiene, periodontal and dental status, but no differences related to age. This might be due to the physical condition not yet affected by metabolic diseases.

A particularity of the expression of AGEs is their increase in hyperglycemic conditions such as diabetes. This disease is strongly associated with a negative impact on soft and hard tissue tooth support, manifesting through periodontitis. Salivary AGEs determinations using nuclear magnetic resonance spectra in a case–control study showed a strong association between the evolution of periodontal disease and AGEs accumulation, evidencing the implication of glycation products in oral diabetes complications [114]. AGEs spectrofluorometry was measured in the saliva, serum, urine and skin in 52 patients with Type 2 diabetes [115]. AGEs accumulation in the skin was strongly age-related and changed in failure of renal function. Renal impairment might lead to the transfer of AGEs to other structures, such as skin.

Wautier *et al.* found that CML blood levels were increased in patients with diabetes versus control group [116]. CML detection in saliva could represent an easy way of assessment of metainflammation in general diseases using a cavitas electrochemical sensor. Local infection found in oral cavity (such as periodontitis and other infection) must be excluded in order to have a correct interpretation of the results [117]. These noninvasive approaches of AGEs assessment in saliva for detection and monitoring general diseases open new and future research areas for real-time detection using wireless transmission.

AGEs in general diseases: *quo vadis*?

The results of *in vitro* and *in vivo* studies vary widely concerning the AGEs levels in biological fluids. These differences are influenced by many external and internal factors. The Western diet, vicious habits, age, sun overexposure increase and accelerate the production and deposition of external AGEs. The process is time and dose dependent, but there is no consensus as to when to start assessing AGEs levels. Should it be done in early childhood, when the body is theoretically free of deleterious substances, and should the obtained measurements be used as a gold standard, or in early adulthood in healthy patients? Systemic diseases, by altering immune host response, activation of proinflammatory mediators, create a favorable environment for the production of AGEs. Moreover, hyperglycemia, hyperlipidemic conditions such as diabetes, dyslipidemia and liver steatosis provide an abundant support for glycation product outcome, and thus, higher values/false-positive values. This is why basic biochemical serum screening is important and should be evermore personalized. Urinary assessments might be influenced by an alteration of renal function, and AGEs might be redirected to other fluids or tissues. From the oral cavity, salivary AGEs might be collected using a cotton swab, in a routine dental examination, the results of which can offer valuable information. Oral cavity pathology could influence the values of salivary AGEs [118].

One of the reasons for which the assessment of AGEs is reliable is their chemical stability, once the Maillard reactions are completed. Even if a large amount is lost by urinary excretion, about 10% is identified in biological fluids and tissues. Animal and human studies have associated the amount of AGEs with physiological and pathological conditions. Among the tested sources, salivary samples are easy to collect and noninvasive, and create a comfortable alternative for the patient. However, protocols that involve providing a sterile environment, transport and examination create time and cost inconveniences.

AGEs downregulation strategies

The source, body localization, ratio and last but not least, the presence of associated diseases has a great influence on the AGE treatment outcome. The current direction is to intervene in the glycation process at key points along its production mechanism, targeting specific pathways and receptors. One direction could be the use of antioxidant drugs. It has been reported that Ca^{2+} channel inhibitors, vitamins thiamine, P (flavonoids) and numerous natural compounds could help in the reduction of AGEs accumulation due to the antioxidant effect [119]. Experimental treatment with monoclonal anti-RAGE IgG3 resulted in improved glucose tolerance and attenuated renal complications in patients with Type 2 diabetes [120]. The influence of local drug delivery such as chitosan-based pH-responsive hydrogels loaded with N-phenacylthiazoliumbromide (PTB) in periodontopathic versus nonperiodontopathic groups was assessed by microcomputer tomography and histology. Results showed that PTB application could slow down the initiation of and improve recovery from experimental periodontitis [121]. It has been demonstrated that the association of antimicrobial doxycycline and ketone compounds such as flavonoids decreases AGE concentration in the crevicular fluid in periodontal disease [122]. Flavonoids inhibit ROS, NO, cyclooxygenase, lipoxygenase and arachidonic acid pathways, which explain their role in reducing AGEs. One mechanism is the activation of dicarbonyl detoxification system, formed by glyoxalase I and II, enzymes which convert AGEs into hydroxyl acids [123]. The other mechanism is the increase of glyoxalase activity with lipoic acid, NAC, thiol-based antioxidants and the subsequent downregulation of glycation product formation [124]. Trans-resveratrol and hesperetin polyphenols activate the glyoxalase system and lead to elimination of MG, property which is active both in normal body conditions – 36° to 37°C and at high temperature during cooking [125]. Some dipeptide compounds – creatine and carnosine, have a carbonyl-trapping effect, and thus reduce the formation and deposition of AGEs. Drinks such as red wine, green tea and various herbal extracts also have antiglycative effects [126]. Innovative approaches act on HMGB-1, WNT-1, S-100 RAGE (which have specificity in osteosarcoma) signaling pathways, and AGE–RAGE interactions. Guilbaud *et al.* suggested that a diet restriction in exogenous AGEs, but rich in purified nutrients, such as vitamins and natural antioxidants could limit the accumulation of glycation products [35]. It seems that diet restriction lowers early glycation products but not the advanced ones. Sell *et al.* measured furosine in skin collagen, and his results suggested that formation of CML and pentosidine depended not only on glycemia but also on oxidative stress and lipid oxidation [127]. A recent study showed that a diet which includes more than 100 g of fructose daily is associated with weight increase among the patients [128]. Another study of Wang *et al.* showed that more than 200 g per day fructose along with a hypercaloric diet led to increased uric acid serum levels [129]. Bunn and Higgins investigated the reactions between glucose, fructose and proteins. Their results showed that fructose is seven-times more reactive to produce Schiff bases, compared with glucose [130]. A meta-analysis study of Cozma *et al.* showed that the replacement of high glycemic index carbohydrates with fructose led to an improvement of glycemic index in diabetic patients, but more than 60 g fructose per day led to serum triglycerides increase, and a total cholesterol lowering [131,132]. The above results state the negative influence of dietary intake in producing glycation compounds.

Due to their benefic effects, carnosine and creatine have been proposed as anti-AGEs strategies; creatine has the ability to block dicarbonyl compounds [133], and carnosine has antioxidant, anti-inflammatory and dicarbonyl-traping effects [134]. Deo *et al.* studied the effect of weight loss on CML and HbA1c serum levels, as glycation markers, in diabetic and nondiabetic patients. Their results showed 17% decrease in serum CML and reduction of HbA1c from 6.8 to 6.2% [135].

All these studies suggest that cautious food cooking (raw aliments, lower temperature and prolonged time), limited carbohydrate intake and natural antioxidants supplements could decrease the AGEs production.

Physical activity & DAGEs

The first studies analyzing the effect of physical activity on glycation compounds were in Malaysian volunteers [136]. After 3 months of twice-per-week tai chi, serum AGEs and malondialdehyde decreased significantly. Another study

analyzed MG in erythrocytes after short and long run. The red cell MG decreased up to 60% in trained men versus 41% in untrained men [137]. A study that investigated endurance running with AGEs – pentosidine deposits in patellar tendons in four groups (elder master athletes vs untrained elder with the average age of 64 years-old and young endurance runners vs untrained young men) found a 21% lower pentosidine density in athletes compared with untrained elderly [138]. Beside the results concerning AGEs, the thickness of the patellar tendons was higher in both elder and young trained men. The researchers suggested that long-term endurance training lowers the age-depending AGEs accumulation. A similar study compared circulating GO, MG and 3-deoxyglucosone, serum CML, CEL, MG-H1 and pentosidine in elder endurance men versus untrained men [139]. The results showed lower MG, 3-deoxyglucosone and MG-H1 concentration in elder athletes. An unexpected result was the concentration of CEL and CML, which were higher in athletes, and directly correlated with cardiorespiratory fitness. Macías-Cervantes *et al.* studied the effect of physical activity (three-times per-week) on overweight men and reduced AGEs intake versus normal food intake for 3 months. AGEs decreased up to 50%, along with the increase of high-density lipoproteins [140]. A prospective study on 98 volunteers investigated the effect of physical activity on circulating sRAGE (known to have an anti-inflammatory efficacy) – measured at baseline, 2, 6 and 8 months [141]. Increase of 9–22% of sRAGE were noticed after sport performing, more obvious in volunteers with initial low performance. The above presented results suggest that physical training could lower AGEs depositing, and a long-term sport practice could have an antiglycative effect and a protective role in general diseases.

Conclusion

Systemic diseases, by altering the immune host response and by activating the proinflammatory mediators, create a favorable environment for the production of AGEs. The Western diet, vicious habits, age and sun overexposure increase and accelerate the production and deposition of AGEs, in a time- and dose-dependent manner. AGEs have a dose- and time-related cumulative negative effect on human tissues. Current anti-AGEs strategies include diet changes either modulation in food cooking, a physical active lifestyle, to increase antioxidative mechanism, and last but not least medication that interfere with the glycation process. Due to their stability in biological fluids, the validation of AGEs as biomarkers, including salivary AGEs, could represent a new approach in the early diagnosis and treatment of diet-related diseases, designing a long-lasting and efficient therapy.

Future perspective

Due to their stability in biological fluids, the validation of AGEs as biomarkers in general diseases by means of accuracy would not be an impediment. In what concerns clinical results, summarized data show inhomogeneous AGE values in the tested samples. This might suggest that a greater number of subjects are required for *in vivo* study in order to achieve a statistically and clinically valid value of this metabolic biomarker. Over time, salivary biosensors have been developed for several applications, including glucose evaluation, stress biomarkers and cortisol detection, salivary amylase and lactate activity, to name a few. However, to the best of our knowledge, there is no device produced and validated to measure salivary AGEs currently used in clinical settings, these were tested only for the research purposes. To assess AGEs real-time variations, the biosensors would be attached to a mouth guard. The device would allow continuous salivary AGEs analysis. This noninvasive evaluation technique could serve for dental office or home management of healthy individuals and general pathology-diseased patients. The noninvasive approaches of AGEs assessment in saliva open new and future research areas for real-time detection using wireless transmission.

Author contributions

AM Băbţan and A Ilea equally conceived and designed the study; contributed to the acquisition; analysis and interpretation of the paper; drafted and critically revised the manuscript. They gave the final approval and they agreed to be accountable for all aspects of the work ensuring integrity and accuracy. BA Boşca, M Crişan and NB Petrescu contributed to the conception and design of the study and the literature search; they drafted and critically revised the manuscript. M Collino, RM Sainz, JQ Gerlach and RS Câmpian contributed to article's design, critically revised, proof reading and gave their final approval concerning the integrity and accuracy of the paper.

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Executive summary

Advanced glycation end products formation

- Advanced glycation end products (AGEs) are represented by structures formed via glycation – a nonenzymatic reaction between sugars with a free amino or ketone group and proteins or lipids, and increase the food attractiveness for intake. Glycation and high-temperature cooking produce stable connections, which make AGEs hard to be countervailed by proteolytic enzymes, antioxidative substances.

AGEs pathogenic pathways downregulation

- AGEs can damage tissues by two different pathogenic pathways: receptor-mediated and receptor-independent manner. The current direction for therapies development is to intervene in the glycation process at key points along its production mechanism, targeting specific pathways and receptors. One direction could be the use of antioxidant drugs. Cautious in food cooking (raw aliments, lower temperature and prolonged time), limited carbohydrate intake and natural antioxidants supplements could decrease the AGEs production. Some results suggest that physical training could lower AGEs depositing, and a long-term sport practice could have an antiglycative effect and a protective role in general diseases.

AGEs in the aging process

- AGEs are considered to be a biomarker for aging, due to the continuous accumulation in body fluids and tissues: in collagen and elastin fibers and in the extracellular matrix, affecting its elasticity. In hard tissues, such as bone, they increase fracture risk.

AGEs in metabolic & neurodegenerative pathology

- A hypercaloric diet not only induces obesity but also influences the cognitive function and memory. Several studies demonstrated the connection between dietary AGEs and neurodegenerative Alzheimer, Parkinson's disease and multiple sclerosis. In inflammatory stress, the CNS reacts by gliosis, a nonspecific hypertrophy of astrocytes, oligodendrocytes and microglia, viewed as protective feedback.

AGEs features & implication in carcinogenesis

- AGEs react with the -NH_2 group of nucleic proteins, ROS, carbonyl stress and could increase the risk of cancer. The cigarette smoke, RAGE polymorphism and AGEs SNP are strongly involved in the development of oral squamous cell carcinoma, melanoma, lung and breast carcinoma.

Salivary AGEs: correspondence with biological fluids AGEs & their appropriateness as biomarkers

- For the noninvasive diagnosis and monitoring of local and general diseases, saliva, as a biological fluid, is very accessible and easy to collect. Repeated samples can be collected, and diagnosis and monitoring using this method is an innovative and attractive approach. To assess AGEs real-time variations, the biosensors would be attached to a mouth guard. The device would allow continuous salivary AGEs analysis. This noninvasive evaluation technique could serve to dental office either home management of healthy and general pathology-diseased patients.

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