DFG-SKLM round table expert meeting

Dietary glycation compounds – implications for human health?

14th of November, 2019

Leibniz Research Centre for Working Environment and Human Factors - Dortmund

Round Table meeting "Dietary glycation compounds –implications for human health?"

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Points to be addressed in the discussion:

Publication: Quality Criteria for Studies on Dietary Glycation Compounds and Human Health

Opinion of the Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG)

Michael Hellwig, Hans-Ulrich Humpf, Jan Hengstler, Angela Mally, Stefan Vieths and Thomas Henle

Check for quality criteria:

- Well-defined structures (Are the structures of glycation compounds well-defined?)

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- Structure-activity approach (Could the observed effects be traced back to defined structures?)

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- Plausibility check of models (Are the structures/dose ranges relevant for human exposure?)

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- Exposure assessment: Quantitative data resulting from state-of-the-art analytical tools and/or validated biomarkers?

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Gaps in knowledge and research needs:

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Session 1

Glycation reactions in food, dietary exposure, uptake and metabolism

Glycation reactions in food: Structures, pathways, quantitative considerations and dietary exposure

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The Maillard reaction (non-enzymatic browning, glycation) is of outstanding importance for the sensory and nutritional quality of processed foods. During the “early stage” of the complex reaction, mainly the e-amino groups of lysine residues react with reducing carbohydrates such as glucose, maltose or lactose to peptide-bound aminoketoses, the so-called Amadori products (e.g. N-□-fructosyllysine, N-□-maltulosyllysine or N-□-lactulosyllysine). These “sugar-amino acids” are degraded during prolonged heating or storage to form 1,2-dicarbonyl compounds (e.g. 3-deoxyglucosulose, 3-DG or methylglyoxal, MGO), which in turn can attack nucleophilic amino acid side chains to form peptide-bound glycation compounds (often referred to as “advanced glycation end products). Besides lysine derivatives (e.g. N-□-carboxymethyllysine, pyrraline, formyline, maltosine) also arginine derivatives (e.g. MG-H1, methylglyoxal-derived hydroimidazolone) and crosslink-amino acids (e.g. pentosidine) are formed. Throughout the last years, we were able to carry out numerous studies on the determination of individual peptide-bound glycation compounds as well as of 1,2-dicarbonyl compounds in a large variety of foods, based on the use of chromatographic techniques and chemically clearly characterized reference material. We have set up an open-access database to collect our own data as well as data from literature and make it available to the scientific community (1).

As part of a conventional diet, daily uptake of glycation compounds is approx. 500-1000 mg Amadori products, 25-75 mg “AGEs” (in particular pyrraline and N-□-carboxymethyllysine), and daily about 1000 mg per day. For 1,2-dicarbonyl compounds, dietary intake was estimated to range between 20 and 160 mg/day for 3-DG and 5 and 20 mg/day for MGO, respectively.

Relatively little is known about bioavailability and the “metabolic transit” of dietary glycation compounds. The Amadori products of lysine are almost completely released by digestive enzymes from heat-treated proteins in short-chain peptides, but these are not absorbed by the body, so that Amadori products cannot be used as a source of lysine ("blocked lysine"). Depending on the heat treatment, most of the lysine in thermally processed foods (e.g. baked goods) may be present in a form that is not biologically available. Thus, depending on the heat

treatment, most of the lysine in thermally processed foods (e.g. bakery products) may be present in a form that is not biologically available. Pyrraline, on the other hand, which is supplied through food, appears almost completely in the urine, making this glycation compound to a perfect marker for the dietary uptake of glycated proteins.

These differences in bioavailability are probably due to the fact that amino acid transporters cannot transport glycated amino acids. Current studies confirm that the nontransportable Amadori products can be rapidly degraded by the intestinal microbiota. Peptide-bound Maillard products (e.g. pyrraline) released in the small intestine are partly substrates of the intestinal peptide transporter PEPT1. For 1,2-dicarbonyl compounds, it was shown that dietary MGO cannot be found in urine, probably due to fast degradation reactions during the gastrointestinal transit. Dietary application of 3-DG, on the other hand, is followed be an increase of 3-DG and its metabolites 3-deoxyfructose (3-DF) and 2-keto-3-deoxygluconic acid (3-DGA), indicating specific mechanisms of biotransformation.

To clearly demonstrate whether dietary glycation products play a role for human health, it should be mandatory to use chemically exact terminology, unambiguously characterized substances, and quantitative data resulting from state-of-the-art analytical tools.

(1) https://lemchem.file3.wcms.tu-dresden.de/

Uptake and metabolism of dietary glycation compounds – Insight from animal studies.

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Most of glycation compounds found in food are considered as non-physiological amino acids that cannot be used as amino acid sources after digestion of proteins. It has long been considered that glycated amino acids are not available for the anabolism of new proteins *in vivo,* regardless of their origin (lysine, arginine or other amino acids). With this in mind, PA Finot et al. (1) created the term "blocked lysine" to describe the lysines which are chemically modified during food processing (mainly by glycation) and which are, in consequence, considered as unavailable for the organism.

Nowadays no one could claim that glycation compounds are not partially metabolized in order to regenerate native amino acids. Insufficient research has been done so far on the topic of metabolization.

In the last decades most of the research has been directed towards the digestion, uptake and elimination of dietary glycation compounds. The only promising data related to the metabolization of dietary glycation compounds come in studies on colonic microbiota (2).

Different approaches have been used to study the digestion, the intestinal absorption and the clearance of the dietary glycation compounds. Animal experiments have been used to follow the metabolic transit of different glycation products administered by oral or injectable routes, and cell cultures have been more specifically used to identify the pathways through which glycated amino acids are transported from the gut to the circulation (3), and from the circulation to the urine. In addition, some clinical studies have been successfully used to complete this research of the metabolic fate of glycation products.

Digestibility of glycated proteins

The resistance of glycated proteins to digestive enzymes is a commonly shared observation from scientists who have tested different forms of such proteins in *in vitro* digestive systems. The apparent low digestibility of glycated proteins may seem quite logical since some of the most important digestive enzymes cleave proteins only between specific native amino acid residues, and are inefficient when glycated amino acids residues are concerned. However animal studies do not always confirm this observation of a lower digestibility of glycated proteins. Our group did not find any difference, for instance, in protein digestibility in rats, between two diets made of extruded and non-extruded caseins, despite a significantly higher level of glycation products in the extruded diet (4). Our observation is consistent with that of another study which used a pig model (5). The authors of the latter concluded that the nutritional consequences of the Maillard reaction in milk was only a decrease in digestibility estimated around 2%. Human studies also concluded that diets high in glycation products only slightly decreased the digestibility of proteins (6)

The observation of a quasi-normal rate of digestibility of glycated proteins does not indicate that all glycation products are digested and well absorbed in the upper intestinal tract. If we consider the most advanced glycation products, the melanoidins, there is no doubt that this group is more resistant to digestive enzymes. Most melanoidins reach the lower gastrointestinal tract unaffected and get partly

digested by the gut microbiota. Although classified as “non-bioavailable glycation products”, melanoidins are at least partly and indirectly available as a result of their microbial metabolization (7).

Bioavailability of dietary advanced glycation end-products

It is commonly presented in the literature that approximately 10 to 30% of dietary advanced glycation end-products (dAGEs) are absorbed into the circulation, and that only 30% of this are eliminated in the urine. This estimation is mainly based on the human study of Koschinsky et al. (8) and the data of Faist and Erbersdobler (9). One clinical trial focusing on the absorption and elimination of pyrraline, Amadori product, and pentosidine demonstrated that the urinary excretion of these three glycation products was more or less dependent on the amounts present in food (10). This study also indicated that each glycation product has a unique fate of absorption/elimination. It also raised the question of the dual origin of some glycation products in urine, exogenous (*i.e.* from food) and endogenous (*i.e. in vivo* glycation), and the difficulties of correctly assessing the part in the urine that comes from food.

In one of his last publications, Finot (11) methodologically presented the difference of absorption and metabolism between free and protein-bound glycation products. The scientific community discovered that this state of glycation product (free or protein bound) was not only important as far as the absorption of glycation products was concerned but also when their concentration in the circulation, tissues and urine were interpreted.

As already mentioned above, each dietary glycation compound has its own bioavailability. Delgado-Andrade and Fogliano have summarized the bioavailability of the Maillard reaction products that are studied the most, in a recent review (7).

We and other groups have focused on carboxymethyllysine (CML) as a model of glycation products although we are aware that every glycation product differs chemically and metabolically. It must be emphasized then that all the results found on CML cannot be extrapolated to every glycation product ingested.

CML was selected not only because it was considered as a good model, but also because of its relatively high abundance in foods and its potential activity after ingestion.

Intestinal transport of CML and other glycated amino acids

It was observed that diets high in CML and some other glycation compounds (but not all of them) lead to an elevation of the same compounds in urine in proportion to the amount ingested (10, 12, 13) This simple observation made both in animals and humans indicates that at least one part of CML is absorbed and taken into the circulation. But by which mechanism glycation products pass the intestinal epithelial barrier and reach the systemic circulation? Most of the work done to answer this question was performed by the team of Thomas Henle. Using caco-2 cell monolayers, T. Henle and his colleagues found that the AGE absorption *in vitro* can take place in the form of dipeptides, but not as free glycated amino acid, most likely by the intestinal proton-coupled peptide transporter (PEPT1) (3). After intercellular peptide bound hydrolysis, glycated amino acids can be released in the basolateral compartments of transwells (approximate equivalent of the systemic circulation) by simple diffusion. This simple way of transport promotes the release of hydrophobic glycated amino acids such as pyrraline, and hypothetically limit the release of hydrophilic ones such as CML.

The same question of transport of glycated amino acids and other AGEs remain unanswered when cells of different organs (liver, kidney, lungs, brain…) are concerned. Do free dAGEs get into cells from different organs? And if so, which transport system is used?

Association between dietary intake and serum CML: why do we have conflicting data in the literature?

Using a specific analytical chromatographic method allowing the quantification of free and protein-bound CML (4), we found recently on an animal model that only plasma free CML increases quickly after an oral intake of CML but not protein-bound CML which remained stable and unaffected by the diet.

We also discovered that the plasma free CML returns quite rapidly to a 'baseline' level after the consumption of a meal. This observation explains why the total CML level (free CML at a baseline level and protein-bound CML level independent of the diet) in blood could appear to be unaffected by the diet when fasting venous blood samples are collected and analyzed.

Despite our limited knowledge about the pharmacokinetics of dCML, our preliminary data indicate that it is characterized by a partial but rapid absorption and elimination. Our findings clearly showed that only the free CML level increased in the animal blood while the protein-bound CML concentration remained unchanged.

It is possible that the apparent low level (14), or lack (15) of association between dCML and serum CML could be accounted for by the fact that most scientists, including ourselves at the time, were performing the analysis on fasting blood samples instead of postprandial blood samples and have, in addition, quantified protein-bound CML rather than free CML.

It is only, however, the protein-bound CML that can be detected and thus quantified when isolated serum proteins are analysed (compared to full serum samples) by chromatographic means or when immunological means (ELISA) are used. We therefore recommend applying Karachalias et al. protocol (16) or our recently published one (4) which both include an LC-MS analysis in order to quantify free CML in blood and to detect any effect of dietary intake.

We also believe, on the basis of the existing literature and our own studies, that only the meals consumed within 24h before the test will significantly affect the level of free CML in blood of healthy subjects. We assume that this last consideration is one more reason, in addition to the lack of free CML analysis and the collection of fasting blood sample, why no association was discovered between dietary intake and serum CML by Semba et al. (15) despite a rigorous dietary assessment taking place on 6 separate days, but not only on the day preceding the blood test.

CML elimination in feces and urine

The most recent data come from the analysis of CML in faeces and urine after well-controlled oral exposures to CML in both animal (17) and human studies (18). Three fundamental conclusions can be drawn from these studies: 1- a very good relationship between dCML and its faecal and urinary excretions; 2- a higher percentage of faecal excretion (22 to 48%) compared to the urinary excretion (7 to 38%); 3- an apparent saturation of urinary excretion when the exposure to CML is increased.

All of the studies that address the elimination of dCML show an apparent incomplete removal of CML in the urine and faeces (no more than 86% in rats and 47% in humans). A hypothetical metabolic fate of CML and a possible retention in some organs and tissues can explain this partial recovery.

To examine this last issue a few recent studies have followed the absorption and the metabolic usage of radiolabeled CML in rodents. While the studies using 18F-fluorobenzoated CML are questionable because of the high chemical modification of the CML (19, 20), the one which used the 14C-labelled CML (21) brought some interesting new elements. The study has confirmed the good bioavailability of CML and its excretion as free CML. However contrary to what we observed the quantification of the radioactivity indicated that more 14C-CML and its unknown 14C-metabolites were eliminated in the urine compared to the faeces. The most important information was that almost 30% of the radioactivity was not recovered the urine, faeces and selected organs leaving the hypothesis of an accumulation in vivo open to question.

With the use of three CML isotopes with different mass-to-charge ratios we were recently able to observe the accumulation of dCML in several organs of mice fed for 30 days with either a control, or a BSA-bound dCML-enriched diet (22). Mice exposed to dCML showed an accumulation in all tissues and organs tested except fat. The rate of deposition was high in kidneys, intestine, and lungs.

However we still do not know how dCML accumulates in tissues and organs. Does it accumulate in cells or does is remain in the extracellular matrices of tissues? The only data that we have so far indicates that the accumulation is not mediated by the receptor for AGE (RAGE).

Apart from the fraction of dCML that stays in organs, what could explain the incomplete elimination of dCML? In part, the answer may lie in the very recent work of M Hellwig and his colleagues (2). They discovered that CML can be metabolized into biogenic amines (i.e. carboxymethyl-derivatives) by different *Escherichia coli* stains *in vitro*. Although this needs to be proved also to be the case in a complex organism, the discovery of three specific metabolites of CML will soon shed new light on the metabolic fate of CML and other dAGEs. The final questions will be: are the metabolites biologically active on the gut microbiota? And can they enter the circulation and have a systemic positive or negative effect on health?

Closing remarks

In conclusion, dietary glycation compounds represent a heterogeneous group of molecules with diverse sensitivities to digestion, various bioavailabilities and unique biological activities. Therefore it would be highly speculative to extrapolate the results obtained from one single AGE and apply them to all the rest.

However the research on a single AGE, such as CML, can be useful for the implementation of new research protocols on other AGEs. We definitely have to learn from ours and others' mistakes, and also from the successes of the past. Progress in the field of glycation can be made only with a full comprehension of the literature and the understanding of the limits of each experiment.

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Dietary glycation compounds in humans and the role of the microbiota

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Glycation compounds have been quantitated in food items such as milk and bakery products, honey, pasta, and malt. Amadori products (e.g., fructosyllysine) are taken up with the daily diet in amounts of up to 1000 mg, while 25-75 mg of glycated amino acids from the late stage of the Maillard reaction (e.g., pyrraline, carboxymethyllysine (CML), and maltosine) are ingested daily.
When ingested with the normal human diet, glycated amino acids are released from food proteins, but only a small proportion is transported into the circulation. The greater part of glycation compounds is transferred into the colon where they are subject to microbial fermentation. Strong interindividual differences were observed between gut microbiota inocula of different human subjects in their ability to degrade CML.[1] Investigations using probiotic *E. coli* model strains revealed the first bacterial metabolites of glycated amino acids: the biogenic amine N-carboxymethylcadaverine (CM-CAD), and the fatty acid N-carboxymethylaminopentanoic acid (CM-APA).[2]

Moreover, glycated amino acids from malt are potential substrates of brewer's yeast *Saccharomyces cerevisiae* during the brewing process. Investigations on the use of glycated amino acids by *S. cerevisiae* in a model system led to the identification of higher alcohols and α-hydroxy acids derived from the Ehrlich pathway as the first known fungal metabolites of glycated amino acids.[3] The novel higher alcohols pyrralinol (up to 200 µg/L) and formylinol (up to 50 µg/L) were quantitated in beer for the first time. The concentrations were particularly high in wheat beer.[4]

The presentation will focus on the latest research on the impact of glycation compounds on microorganisms as fermentable substrates, sources of possible bioactive metabolites and signalling molecules.

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Session 2

Dietary glycation compounds and biological effects

Dietary glycation compounds and physiological consequences

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The term dietary advanced glycation end-products (dAGEs) referrers chemical structures such as carboxymethyl-lysine, fructosyl-lysine, pyrraline, or pentosidine, which are present in thermally processed food both bound to protein (on the side residues of Lys and Arg) and in free form, derived from the reaction with free amino acids. The singularity of these compounds is that they seem to have an additive effect with *in vivo* generated AGEs, giving rise to detrimental physiological consequences related to the advance and progress of different degenerative disorders. Several evidence of pro-inflammatory action, impairment of glucose management and progression of mental degenerative disease has been found in animal and humans. However, the information must be handle with care since most of the *in vivo* intervention trials could be biased by different reasons. A direct association between the well-known damages induced by the Western diet and the intake of dAGEs might be an oversimplification, since many studies ignore lipid oxidation or calorie density of foods as significant factors. The current challenge for nutrition, food technology and biomedical professionals is the design of studies able to disentangle the presence of dAGEs from other common features of heavily processed, calorie dense food.

Adverse effects of dietary glycation compounds.

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Dietary “advanced glycation end products” (“AGEs”) are increasingly reported to be linked to adverse health effects in humans, including food allergy, diabetes mellitus, chronic inflammation and cardiovascular disease, Alzheimer's disease, and cancer.

The term “AGEs” is widely used to summarize a wide range of structurally diverse compounds that derive from the Maillard reaction. It comprises (1) Amadori compounds (e.g. N-ε-fructoselysin) that are formed in the early stage of the Maillard reaction from reaction of reducing sugars with free or peptide-bound amino acids, (2) dicarbonyl compounds (e.g. glyoxal) formed by degradation of Amadori products during advanced stages of the Maillard reaction, (3) glycated peptide-bound amino acid derivatives that result from reaction of dicarbonyl componds with the ε-amino group of lysine or the guanidino group of arginine (e.g. carboxymethylysine), as well as (4) stable end products (e.g. 5-hydroxymethylfurfural, acrylamide).

Considering the structural diversity of compounds formed in food during heating, which includes highly reactive dicarbonyl compounds as well as stable peptide-bound glycated amino acid derivatives, it is evident that their toxicokinetic and toxicodynamic properties may be equally diverse. It follows that assessment of potential health risks associated with dietary intake of AGEs can only be made based on identfication and characterization of the toxicological hazards of individual glycation compounds that humans are exposed to via food.

General considerations Generally, compounds present in food may induce local effects within the gastrointestinal tract and/or potential systemic effects, provided they become bioavailable. Local effects of glycation compounds may include direct toxicity of the compound to the gut epithelium, including potential genotoxicity and mutagenicity, but also interference with the gut microbiome, which is increasingly considered as a contributor to human diseases. There is evidence that the gut microbiota may utilize glycated amino acid derivatives as an energy source and thus glycation compounds may modulate the bacterial gut microflora. On the other hand, the gut microflora may contribute to metabolism and hence not only to biodegradation but potentially also to bioactivation of dietary glycation products. A key question in health risk assessment of dietary glycation compounds that needs to be answered in order to design appropriate toxicity studies is to understand to what extent and in which form dietary glycation compounds are bioavailable. While this may be relatively straight forward for dicarbonyl compounds and stable end products such as hydroxymethylfurfural or acrylamide, there is as yet insufficient knowledge on the digestibility of glycated proteins and absorption of free vs. protein- or peptide-bound amino acid derivatives. A further critical aspects in health risk assessment of dietary glycation compounds is the fact that these may also be formed endogenously, and thus studies are needed to understand the contribution of dietary glycation compounds to overall exposure and adverse effects.

Amadori Compounds. Furosine (ε-N-2-furoylmethyl-L-lysine, FML) is a compound that is not present in food as such but is formed during sample work-up by acid hydrolysis of Amadori compounds in food. Furosine is widely used by food chemists as an important marker of the Maillard reaction. The toxicity of furosine has been studies in vitro and in vivo (Li et al., 2019a; Li et al., 2019b; Li et al., 2018; Saeed et al., 2017). While furosine was not mutagenic in the Ames assay (TA 100 and TA 1535), it was reported to be a strong toxicant, particularly in liver and kidney cells (Saeed et al., 2017). In vivo studies using administration of furosine by intragastric gavage demonstrate that furosine causes injury to mice liver kidney and testicle tissue via induction of apoptosis and inflammatory responses (Li et al., 2019a; Li et al., 2019b; Li et al., 2018). The significance of these findings is unclear as it is not expected that humans are exposed to furosine, which is artificially generated during food analysis.

Dicarbonyl Compounds. Considering their chemical reactivity and thus apparent potential to cause toxicity through covalent binding to cellular macromolecules, dicarbonyl compounds are among the most widely studied glycation compounds. While there is limited data on the absorption and distribution of glyoxal in humans and experimental animals, biotransformation of glyoxal via the cytosolic GSH-dependent glyoxalase system as the major pathway for the detoxification of glyoxal and via 2-ketoaldehyde dehdrogenase is well described. Glyoxal causes irritations to the gastrointestinal tract and degenerative changes in the kidney and pancreas as the main target organs. Glyoxal is clearly genotoxic. It forms stable DNA adducts and DNA cross links, and has been shown to be mutagenic, to induce chromosomal aberrations, DNA repair, and sister chromatid exchanges (Vilanova et al., 2017) (Madhaven, 2000; MAK, 2014; WHO, 2004). Similarly, methylglyoxal is mutagenic in the absence of exogenous metabolic activation in S. typhimurium, E. coli and Saccharomyces cerevisiae, induces sister chromatid exchange, chromosomal aberrations and micronuclei in mammalian cells, and has been shown to form DNA adducts and DNA cross-links (IARC, 1991; Marnett, 1994). There is also evidence that covalent binding of methylgloxal to cellular proteins, i.e. glycation of proteins, such as histones and glyceraldehyde-3-phosphate dehydrogenase (Lee et al., 2005) may alter chromatin structure (Galligan et al., 2018) and Inhibit mitochondrial respiration and glycolysis, respectively, and may thus link protein adduction by methylglyoxal to altered cell function and metabolism. There are however no adequate in vivo studies on the chronic toxicity and carcinogenicity of methylglyoxal (IARC, 1991). Both glyoxal and methylglyoxal are also formed endogenously during normal cellular metabolism by a multitude of enzyme independent pathways (Kalapos, 1999). Similarly, 3-deoxyglucosone is formed endogenously from glucose via the Maillard reaction and polyol pathway (Niwa, 1999). 3-Deoxyglucosone readily reacts with deoxyguanosine (Hayase and Kaneko, 1998); it is mutagenic and induces chromosomal aberrations in vitro. There are only limited data on 3-deoxyglucosone in vivo, which so far suggest a low rate of absorption from the GI tract and rapid excretion in urine, predominantly as 3-deoxyfructose (Kato et al., 1990).

Glycated peptide-bound amino acid derivatives. While covalent binding of reactive intermediates to cellular macromolecules is a well established cause of toxicity through altering biological function of the macromolecule, reaction of dicarbonyl compounds with free or protein bound amino acids in food may be seen primarily as a mechanism of detoxication of these reactive intermediates. However, potential negative effects of protein glycation may include food allergenicity, reduced nutritional quality, and potential interaction of the free and protein-bound modified amino acids with biological targets. It is evident that the toxicokinetic and toxicodynamic properties of free glycated amino acid

derivatives may be substantially different from protein- or peptide-bound amino acid derivatives. For instance, absorption of carboxymethyllysine (CML) from the GI tract depends on the free or protein-bound form and on the matrix. There is also evidence that protein-bound CML may be a ligand to the RAGE receptor (receptor for advanced glycation end products) and subsequently to ellicit an inflammatory response, whereas free CML appears not bind to RAGE. Oral toxicity studies using free CML suggest that prolonged exposure to (free) CML may impair liver and kidney function (Li et al., 2015; Liu et al., 2016), but the molecular mechanisms underlying these effects have not investigated. So far, there very limited to no toxicity data on free or protein-bound glycated amino acid derivatives other than CML, such as formyline and pyrraline.

Stable end products. The Maillard reaction also gives rise to a range of stable end products. An example is 5-hydroxymethylfurfural (5-HMF) present in a wide variety of food items. Although 5-HMF can be bioactivated to a reactive and mutagenic metabolite 5-sulphoxymethylfurfural, the currently available suggest that 5-HMF is not genotoxic and does not induce neoplastic changes in the intestinal tract (Abraham et al., 2011). Acrylamide is a Maillard reaction product that has probably received most attention. Following absorption from the gastrointestinal tract, acrylamide is extensively metabolised, predominantly by conjugation with glutathione but also to glycidamide, which readily reacts with DNA and is considered to be the metabolite responsible for acrylamide genotoxicity and carcinogenicity (EFSA, 2015). Besides carcinogenic effects, studies in experimental animals show that acrylamide may induce neurotoxicity, reproductive and developmental toxicity. In assessing health risks related to acrylamide in food, the European Food Safety Authority (EFSA) concluded that the margins of exposure (MOEs) indicate a concern for neoplastic effects of acrylamide (EFSA, 2015).

Conclusions. The Maillard recation gives rise to a complex mixture of compounds, each with distinct toxicokinetic and toxicodynamic properties and hence potential to cause adverse effects. In order to assess risks to human health related to dietary intake of glycation compounds, it is essential to determine the toxicity of each individual compound or compound class, including a thorough understanding of the fate of compounds upon oral intake. Care must be taken not to confuse free and protein-bound glycated amino acid derivatives. Generation of high quality data as a basis for health risk assessment will require close interdisciplinary cooperation between food chemists and toxicologists.

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Session 3

Endogenous glycation reactions and health

Reactive Metabolites accelerating aging and diabetes-associated complications (SFB 1118 – Reactive metabolites as a cause of diabetic complication)

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Since intensive glucose control was not able to reduce organ complications significantly in patients with diabetes, other metabolic pathways such like reactive metabolites, dicarbonyl stress and ROS moved in focus of current research. Increased dicarbonyl stress and reactive metabolites are associated with diabetes-associated complications by AGE-formation, chronic inflammation, protein cross-linking and DNA-damage. However, not increased glucose flux seems to be responsible for increased dicarbonyl stress, impaired detoxification pathways of reactive metabolites are associated with diabetic phenotype. Interestingly, detoxification is not only depending on Glyoxalase-1 but also on Aldo-Keto-Reductase and Aldehyde-Dehydrogenase which are involved in the development of diabetic complications.
Furthermore, increased DNA-damage is associated with increased reactive metabolites and AGE-formation and seems to be a common soil in diabetic complications. Patients with type 2 diabetes showed increased DNA-damage which is associated with fibrotic organ dysfunction of lung, kidney and liver. First data in STZ-mice indicates that phosphorylated RAGE in the nucleus is related to DNA-repair and could be potential target for remission of complications in the future.

Session 4

Dietary glycation compounds and health I

Dietary glycation compounds and their role in diabetes

DFG-SKLM round table expert meeting; 14th of November 2019

Prof Dr Casper G. Schalkwijk

Several chronic inflammatory diseases have reached “epidemic” proportions over the last 50 years, including type 2 diabetes (T2DM). Current efforts to arrest the epidemic of T2DM have had limited success. Thus, there is an urgent unmet need for effective approaches to prevent the development T2DM. Since it is unlikely that the genetic make-up of humankind has changed significantly in the past 50 years, it is generally accepted that a change in diet is a key determinant of T2DM epidemic. Dietary factors impact inflammation and endothelial function, both affect insulin resistance. Among other factors, advanced glycation endproducts (AGEs) in food are potential risk factors for insulin resistance and T2DM (1).

AGEs are a heterogeneous group of bioactive compounds that are formed in the body when reducing sugars reacts with amino acids in proteins and other macromolecules. Several mechanisms have been proposed by which AGEs may adversely affect human health including, the accumulation of AGEs in the extracellular matrix and activation of key cell signalling pathways. Over de last decade we have gained a substantial amount of data obtained from in vitro work, animal models, and from epidemiological studies, about the role of AGEs in insulin resistance, the development of type 2 diabetes and vascular complications. Our previous research *in vitro* has demonstrated that AGEs are implicated in the pathogenesis of age-related diseases in particular insulin resistance, diabetes, inflammation, and vascular diseases (2-8). In addition, AGEs are direct modulators of β-cell function (9, 10). Moreover, we recently found in db/db mice that the AGE-axis is a key modulator of obesity-induced insulin resistance (11). We demonstrated that a delayed intervention with pyridoxamine, a vitamin B6 analogue with anti-glycating capacity, protected against impaired glucose metabolism and insulin resistance (7). These findings highlight the potential of AGE inhibition to serve as an intervention strategy in insulin resistance. Thus, there is ample evidence for an association between AGEs and insulin resistance and beta-cell function.

In addition to endogenous formation, AGEs are also formed during processing of food. In the Netherlands, as in other developed countries, methods for food processing as well as meal composition have changed. Modern foods contain excess fat and sugar, which in the presence of protein are most susceptible to AGE formation with cooking temperature as a potent promoter (12, 13). It has now become apparent from animal models that dietary AGEs represent a source of circulating and tissue AGEs (14), manifesting similar pathogenic properties to their endogenous counterparts including the induction of insulin resistance and development of diabetes (15).

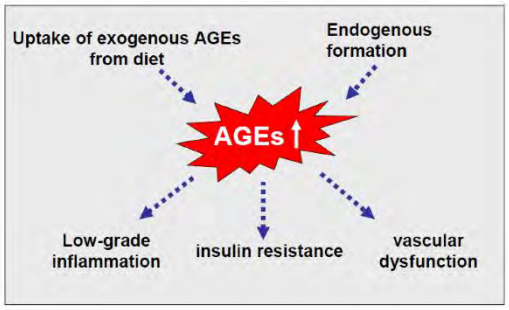

Figure 1. The level of AGEs in the body can be increased via different routes and have an adverse effect on health.

We have developed a new dietary AGEs database of three major AGEs as analyzed in 192 specific food items, based on a state-of-the art UPLC-MSMS technique (16). In the first analysis with this dietary AGE database and food frequency questionnaires in a cohort of 465 participants, we have found that

higher levels of dietary AGEs are associated with free plasma and urinary AGE levels. These findings may have important implications for those who ingest a diet rich in AGEs (17). In an animal model we demonstrated that higher dietary AGE intake results in higher levels of AGEs in several organs including the liver and the brain and increased hepatic inflammation. The accumulation of AGEs in organs was reversible by a switch to low dietary AGE intake.

Although dietary AGEs restriction has been associated with reduction of serum and tissue AGEs in parallel with a reduction of markers of inflammation and endothelial dysfunction, as well as improvement of insulin sensitivity (18-24), it is still unresolved whether dietary AGEs are directly involved in the aetiology of insulin resistance in human (25). In fact, studies so far have yielded inconsistent results, because of poor methodological quality and/or multiple shortcomings:

- Many of the studies so far used controlled variations in cooking methods to increase AGEs in the food. However, the heating of food also induces degradation and oxidation of heat-sensitive nutritive compounds. A high- versus low-AGE diet prepared by different heat treatments will, therefore, lead to dissimilar contents of such nutritive compounds and therefore the effects of high AGE diets cannot be directly related only to AGEs.
- In most studies, an important issue is the limited reliability of immunoassays for the AGEs that are used for quantification.
- Many of the studies did not control for the energy and/or macronutrient content (26, 27). Low- and high-AGE diets have to be similar in energy and nutrient content, as physiological endpoints are influenced by these factors.
- Although a randomised cross-over study found that high AGE intake reduced insulin sensitivity (24), it was difficult to exclude possible carry-over effects; a four-week wash out period might be too short.
- A randomized trial was conducted in two parallel groups; however shortcomings of this trial were the use of HOMA-IR instead of direct assessment of insulin sensitivity and the lack of data about beta-cell function (23).

So far, it remains unresolved whether dietary AGEs are causally involved in the development of insulin resistance and T2DM. To rule out all the above-mentioned research gaps we have decided to conduct a new and unique study. We are now performing a new and unique study: a randomized trial in overweight subjects in two parallel groups. The participants are allocated into either a low-AGE group or a high-AGE group based on our unique dietary AGE database. We are using state-of the art UPLC-MS/MS for the measurement of AGEs. Insulin resistance, as measured with a clamp, is the primary outcome. We are measuring the effect of dietary AGEs on beta-cell function as obtained from an OGT, and will for the first time combine this primary outcome with macro- and microvascular function, and biomarkers of AGEs, endothelial dysfunction and of low-grade inflammation. Last participant out is expected at February 2021.

It is now well established that the dicarbonyl compounds methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG) are the most reactive precursors in the formation of AGEs. The glycation activity of these biologically reactive dicarbonyl compounds is much higher as compared to that of sugars, with MGO as the major precursor in the formation of AGEs (28).

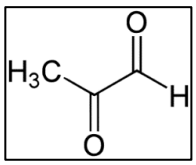

Figure 2. Methylglyoxal

There is now increasing evidence that elevated levels of MGO is at the root of the development of chronic inflammatory diseases (29-31). Studies in experimental models have demonstrated that administration of MGO results in the development of diabetes (32) and MGO also plays a role in the development of other age-related diseases such as neurodegenerative disorders (as reviewed in (33). Building further on these results, we have quantified MGO (and GO and 3DG) in different beverages and food products as analysed with a state-of-the art UPLC-MSMS technique. We found high

concentration of dicarbonyls in sugar- and fat containing foods and developed a dietary dicarbonyl database of MGO, GO and 3-DG. So far, the gastrointestinal absorption, bioavailability and a role in gut microbiota and physiological consequences of dicarbonyl compounds in foods are not well known (34, 35). We recently found in mice that dietary MGO are absorbed from the diet and lead to accumulation of MGO-derived MG-H1 in plasma and in tissues including the brain (preliminary data; see figure).

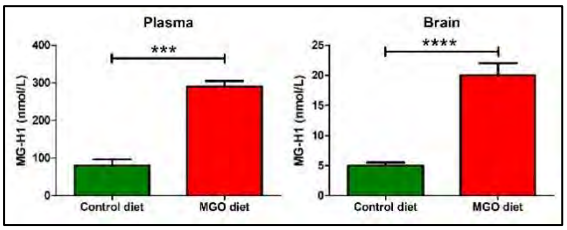

Figure 2. Dietary MGO increases the levels of MGO-derived MG-H1 in plasma and brain in mice (n=6).

MG-H1 is a major MGO-derived AGE and strongly linked to several chronic inflammatory diseases (36). The described dietary dicarbonyl database opens the possibility to further explore the physiological impact of dietary MGO to the organism.

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Glycation and Uremia

Monika Pischetsrieder

Chronic kidney disease and acute renal failure leads to the systemic accumulation of urea and other toxic waste products (uremia). The concentrations of advanced glycation end-products are greatly elevated in the plasma and hemolysate of uremic patients.[1,2,3] These elevated AGE-levels are caused by incomplete clearance, but also by increased formation due to oxidative and carbonyl stress[3,4,5].

The role of carbonyl stress in uremia

Carbonyl stress is characterized by the accumulation of reactive carbonyl compounds in plasma and tissues, which are considerably more active to generate AGEs compared to sugars. In one study, reactive carbonyl compounds generated more than 70 resp. 80 % of the AGEs CML and imidazolone despite of a 500 fold molar excess of glucose[6]. They are formed by the breakdown of sugars and comprise α-dicarbonyl compounds and short chain monocarbonyl compounds, which readily react with amino acid side chains of proteins yielding AGEs. Most prominent reactive carbonyl compounds are 3-desoxyglucoson (3-DG), 3,4-didesoxyglucoson-3-en (3,4-DGE), glucoson, 3-desoxygalactoson (3-DGal), methylglyoxal (MO) and glyoxal (GO) (Figure 1). Additionally non-sugar monocarbonyls can be of relevance, such as 5-hydroxymethylfurfural (5-HMF) or formaldehyde.

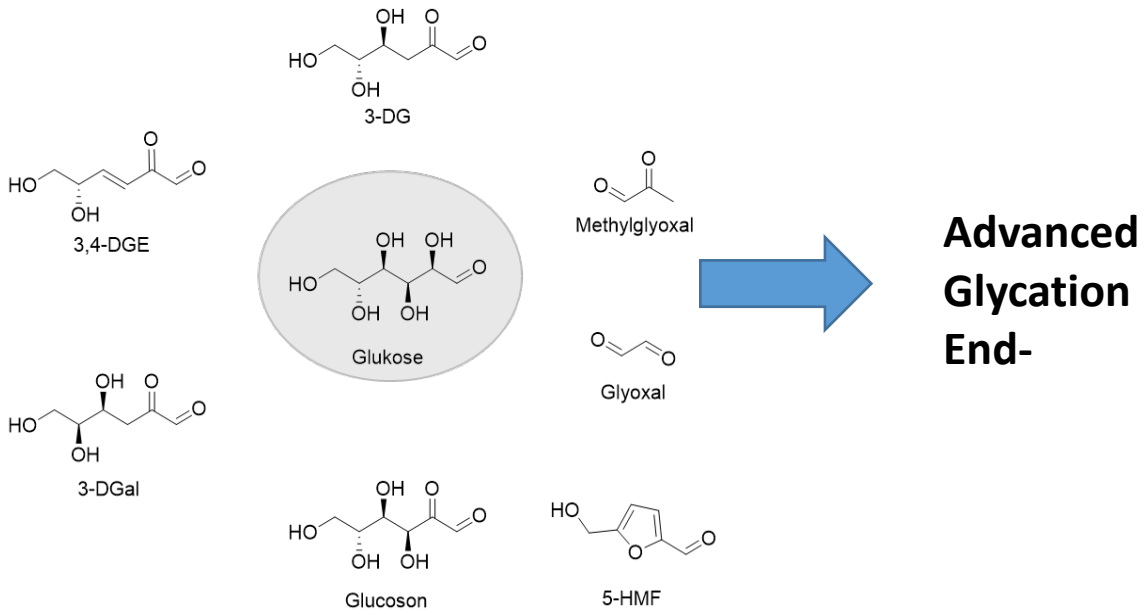

Figure 1: Structures of important reactive carbonyl compounds causing carbonyl stress in chronic kidney disease. Carbonyl stress leads to high levels of AGEs in the plasma and tissues of uremic patients

Carbonyl stress in uremic patients is caused by different mechanisms. In undialyzed patients with chronic renal failure, serum 3-DG levels are fourfold higher compared to healthy control. This increase is partially due to insufficient renal clearance, since 3-DG serum concentrations positively correlate with serum creatinine. Additionally, the main metabolization pathway of 3-DG, the reduction to 3-deoxyfructosone in the kidney may be impaired by the loss of kidney function.[7] Hemodialysis removes 3-

DG strongly, but not completely from the serum, since the levels after hemodialysis are still higher compared to healthy subjects.[7] Similarly, other reactive carbonyl compounds are also elevated in uremia[8]. In contrast to hemodialysis, continuous ambulatory peritoneal dialysis does not sufficiently remove reactive carbonyl compounds, and beyond that is even an additional source. During heat sterilization of glucose containing peritoneal dialysis fluids, reactive carbonyl compounds, the so-called glucose degradation products, are formed, which are permanently administered in high amounts together with a concentrated glucose solution into the peritoneal cavity (Figure 2).[9]

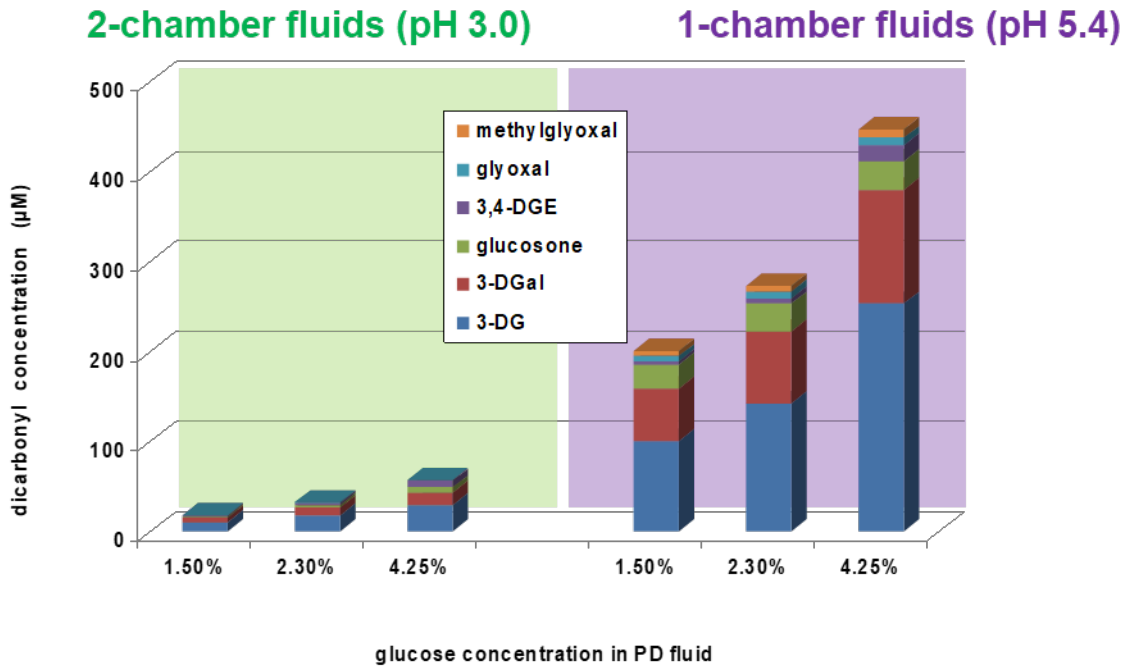

Figure 2: Concentrations of reactive carbonyl compounds (glucose degradation products) in different peritoneal dialysis fluids according to Mittelmaier et al.[10]

Consequences of carbonyl stress in peritoneal dialysis[9]

As a consequence of the permanent carbonyl stress in the peritoneal cavity of peritoneal dialysis (PD) patients, high levels of AGEs are formed locally in the peritoneum. Peritoneal AGE levels are associated with morphological changes, interstitial fibrosis, vascular sclerosis and peritoneal permeability. Eventually these damages lead to a loss of ultrafiltration capacity and therapy discontinuation. Furthermore, glucose degradation products are cytotoxic, leading to mesothelial denudation, which is associated with fibrosis and vasculopathy and thus may be a second cause of ultrafiltration failure. The reactive carbonyl compounds present in PD fluids are not equally harmful. 3,4-DGE is the strongest contributor to the cytotoxic effect of PD fluids, although it is present in only very small amounts compared to other products. 3,4-DGE is a highly reactive compound due to its Michael system which readily reacts with nucleophils, particularly with thiol groups of cysteine residues. AGE formation by PD fluids and resulting loss of enzymatic activity is caused by 3,4-DGE, Glucosone, 3-DGal and 3-DG, whereas methylglyoxal and glyoxal are insignificant contributors (Figure 3).

The systemic effects of reactive carbonyl compounds in PD fluids are not well established. Thus far, it is not clear to which extent these products can penetrate through the peritoneal membrane into the

blood stream. However, there is evidence that 3-DG is absorbed to some extent. Consequently, serum AGE concentrations, in particular those of imidazolone, are lower when patients are treated with fluids with reduced levels of reactive carbonyl compounds. Furthermore, PD patients benefit from the administration of reduced levels by improved residual renal function, lower markers of peritoneal inflammation and higher markers of mesothelial cell mass.

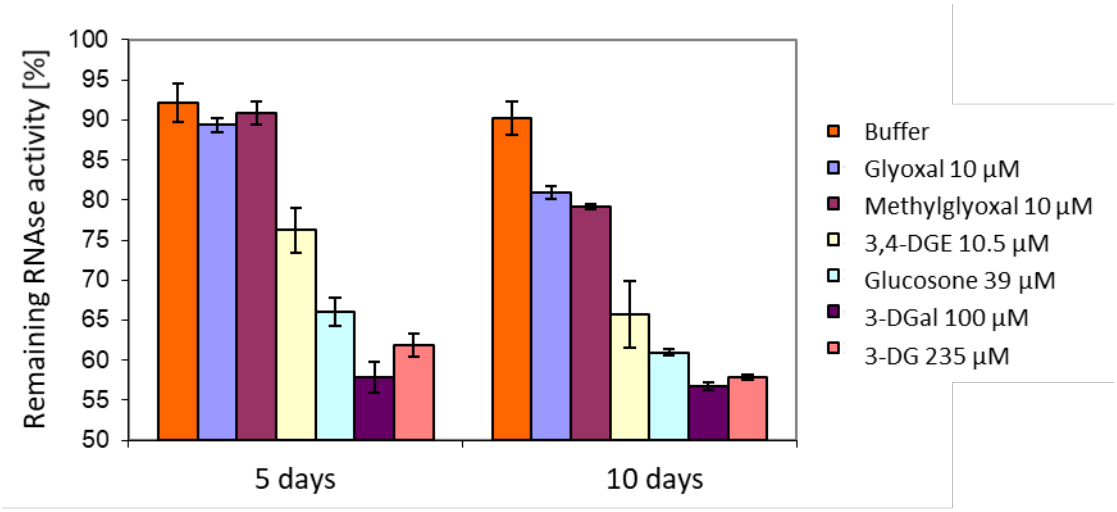

Figure 3: Reactive carbonyl compounds have different glycation activity. RNAse was incubated with different reactive carbonyl compounds in concentrations as present in PD fluids. Glycation of RNAse leads to a loss of enzymatic activity[11]

Carbonyl stress in peritoneal dialysis patients– mitigation strategies[12]

Reactive carbonyl compounds are formed in PD fluids by the breakdown of the osmotic agent glucose during heat sterilization. Glucose degradation is dependent on the several parameters so that the formation rate can be modulated by the heating conditions. For commercial production, however, the variation possibilities are limited because it is mandatory that sterilization is complete and biocompatibility of the final product assured. Feasible mitigation strategies, which are clinically applied, comprise the use of double chamber bags and the replacement of glucose by alternative osmotic agents. Double chamber bags allow the separate sterilization of the electrolyte and the glucose solution, so that the latter can be heated at a low pH value of about 3.0. Glucose degradation is highly dependent on the pH value with a minimum degradation at slightly acidic pH (Figure 2). The physiological pH of the fluid is later obtained by mixing both solution immediately prior to use.

As an alternative, amino acids, and more commonly, polydisperse glucose polymers with a molecular weight between 1,600 and 45,000 Da can replace glucose as an osmotic agent. Glucose polymers possess less reducing ends than glucose. Since these reducing ends are the starting point for the formation of reactive carbonyl compounds, the resulting solutions generate less carbonyl stress to the patients.

Conclusion

Strongly elevated AGE-levels in plasma of patients with chronic kidney disease is caused by a reduced clearance, but also by elevated oxidative and carbonyl stress. Carbonyl stress is a consequence of the disease, but also peritoneal dialysis, which is a commonly applied therapy option, can augment local and systemic carbonyl stress with severe clinical consequences. Fundamental understanding of the chemical mechanisms involved in the formation and reaction of reactive carbonyl compounds is the basis for the development of suitable mitigation strategies.

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Session 5

Dietary glycation compounds and health II

Glycation and aging

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Aging is the progressive accumulation of damage with time leading to cellular as well as to organ dysfunction. This will increase the risk for degenerative diseases as well as for death. Nutrition on the other hand can have a great impact on the development of diseases and / or the maintaining of health. Therefore, the relationship between health, aging, diseases and nutrition has been a subject matter of research.

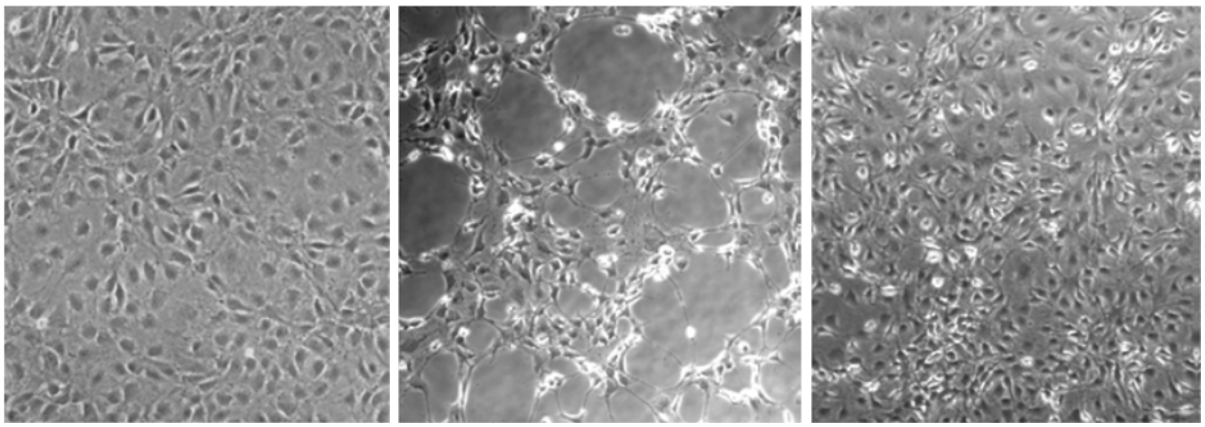
An important example of molecules, bridging nutrition, diseases and aging are the Advanced Glycation Endproducts (AGEs) or Maillard Reaction Products (MRPs), products of the browning reaction. In 1912, Louis Camille Maillard first observed the browning reaction by heating glycine and glucose, which was thereafter referred as the Maillard reaction (1). As a first step of this reaction with proteins, reactive carbohydrates e.g. glucose react with the amino group sidechain of lysine or arginine to form a Schiff base. After a rearrangement, a semi-stable Amadori product – a ketosamine structure – was formed. The best-known example of these reaction products is the glucose-modified hemoglobin – HbA1c – used in medicine as a marker for long-term hyperglycemia in Diabetes (2). Subsequent reactions lead to stable endproducts, the AGEs formed slowly at low temperature (medicine) or the MRPs (nutrition) formed fast at high temperature. Whereas many end-products are the same within AGEs and MRPs (as for example Carboxymethyllysine – CML, methylglyoxal–hydroimidazalone - MG-H1, Carboxy-ethylysine – CEL, or pentosidine), MRPs additionally contain high molecular weight products like the melanoidins.

AGEs are clearly associated with the aging process. It was shown, that glycated collagen accumulate during aging and Diabetes Mellitus (3). As proteins can crosslink during the glycation process, accumulation of cross-linked extracellular matrix proteins will lead to tissue stiffening. This is a hallmark of diastolic heart failure as well as hypertension in the elderly. Glycation of proteins can change/reduce their function. This was shown for hormones and growth factors like insulin and platelet derived growth factor (PDGF) (4). In addition, AGE-modified proteins can bind to the

multiligand receptor RAGE (receptor for advanced glycation endproducts), thereby inducing a proinflammatory response. Within the aging process, it is still unclear to what extend the endogenous or the exogenous AGEs are responsible for these effects. Due to the proposed effects of AGEs on health of mice and men, the group of Helen Vlassara introduced glycotoxins as a name for AGEs and AGE producing substances in food (5).

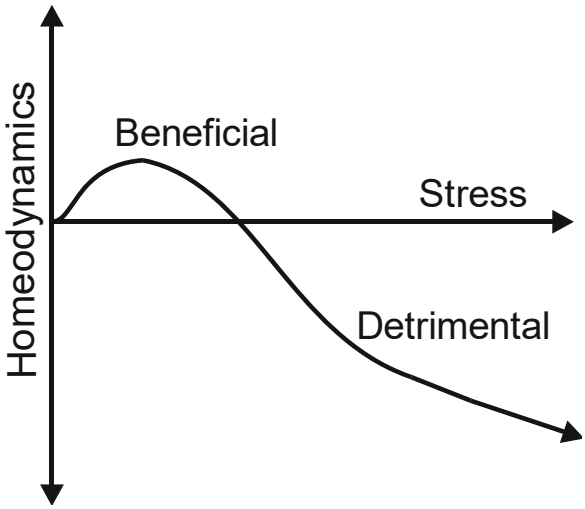
We in contrast could demonstrate a beneficial tumor growth–inhibiting effect of plasma advanced glycation end products as well as of an AGE-rich diet (bread crust) in non–small cell lung carcinoma *in vitro* (cell culture 2D and tumor spheroids 3D) and *in vivo* (6). Patients with lung cancer and high AGE associated fluorescence in the plasma at the time of operation survive better (5 year postoperative survival) in comparison to patients with low levels of AGEs. In addition, tumor growth of human H358 lung carcinoma cells subcutaneously implanted into athymic mice receiving AGE-rich bread crust diet was reduced in comparison to mice with control diet (day 25 of the tumor growth).

In the cardiovascular system, we could further show that water soluble extracts of bread crust activates cardiac cells. In mouse cardiac fibroblasts, bread crust extract induced a moderate elevation of ROS production causing an activation of p42/p44MAPK, p38MAPK and NF-κB, followed by increased expression of anti-oxidative enzymes like the Mn-SOD (7). Pretreatment of these cardiac cells with bread crust extract increases stress tolerance and survival after oxidative stress (24 hours treatment with 0.7mM H_2O_2).

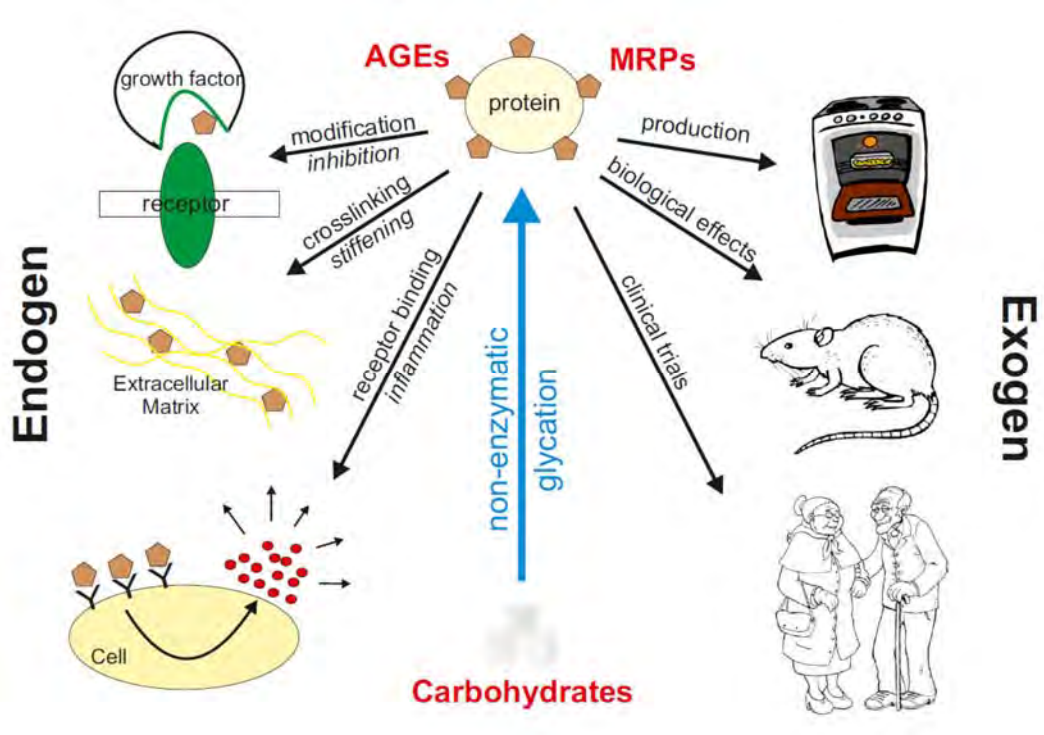


Control cells | H_2O_2 treated cells | H_2O_2 treated after BCE pretreatment

Beside the potential negative effect, AGEs / MRPs from nutrition can have positive effects on health by hormetic responses. AGEs induce intracellular oxidative stress, thereby increasing the endogenous defense systems in the long-term. Like exercise, nutritional AGEs may be protective by inducing limited stress.



This principle is known in aging research as hormesis. Whereas we recognize many delirious effects of AGEs produced endogenously, little is known about food MRPs / AGEs within the aging process. Can we modify the production to have a positive impact on health and aging and what are the important biological effects to focus on? Finally yet importantly, we urgently need real randomized clinical trials in future.



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Glycation and (food) allergy

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The Maillard reaction (MR) leads to modification of proteins with various types of glycation structures. Therefore, the potential impact of the MR on the immunogenicity and potential allergenicity of food proteins has been a matter of concerns. Recent studies suggested that MR products, in particular "advanced glycation end products (AGEs)," as constituents of the diet may be involved in the development of chronic inflammation by acting as inflammatory components and affecting the gut microbiome. Type I allergies are caused by an inadequate immunological response to otherwise harmless environmental proteins from e.g. pollen, insects, or foods. This immune response is characterized as primarily T-helper cell 2 (Th2) type driven, resulting in the formation of allergen-specific Immunoglobulin E (IgE) antibodies and leading to mast cell activation upon secondary contact with the respective allergen. Potential modulatory effects of glycated food proteins in the context of allergy could be (i) creating novel IgE binding epitopes (ii) modulating T cell immunogenicity of food proteins by pathogen recognition receptors (PRR) including macrophage scavenger receptors and the receptor for advanced glycation end products (RAGE) (iii) effects on T cell differentiation promoting Th2 responses (iv) generally enhancing inflammatory conditions and oxidative stress and (v) causing dysbiosis or reduced diversity of the intestinal flora leading enhanced susceptibility to allergies. The current knowledge of the different potential enhancement mechanism is briefly reviewed and own data of model studies using glycated ovalbumin in mouse models and human cell culture experiments are presented. Taken together our data suggest that through their activity via Scavanger receptor A (SR-A) glycation products may cause enhanced allergen uptake by antigen presenting cells, enhanced $CD4^+$ T-cell activation as well as enhanced production of allergen specific IgE, and therefore lead to enhanced allergenicity of highly thermally processed foods. However, this hypothesis needs to be further investigated in clinical studies in patients with confirmed food allergies.

This review found that the biological, immunological, and allergic properties of dietary MR products are diverse due to the complexity of the MR.