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Oxidative stress, protein glycation and nutrition – interactions relevant to health and disease throughout the lifecycle

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Protein glycation has been studied for over a century now and plays an important role in disease pathogenesis throughout the lifecycle. Strongly related to diabetic complications, glycation of Hb has become the gold standard method for diabetes diagnosis and monitoring. It is however attracting attention in normoglycaemia as well lately. Longitudinal studies increasingly suggest a positive relationship between glycation and the risk of chronic diseases in normoglycaemic individuals, but the mechanisms behind this association remain unclear. The interaction between glycation and oxidative stress may be particularly relevant in the normoglycaemic context, as suggested by recent epidemiological and *in vitro* evidence. In that context nutritional and lifestyle factors with an influence on redox status, such as smoking, fruit and vegetable and antioxidants consumption, may have the capacity to promote or inhibit glycation. However, experimental data from controlled trials are lacking the quality and rigour needed to reach firm conclusions. In the present review, we discuss the importance of glycation for health through the lifecycle and focus on the importance of oxidative stress as a driver for glycation. The importance of nutrition to modulate glycation is discussed, based on the evidence available and recommendations towards higher quality future research are made.

Glycation: Oxidative stress: Antioxidant: Nutrition: Diabetes mellitus: Chronic disease: Polyphenols

Glycation reaction: historical background

Glycation, also referred to as non-enzymatic browning or the Maillard reaction, has attracted scientific interest for nearly a century. Initiated by the non-enzymatic condensation of a reducing sugar (such as glucose) with a protein, glycation is one of the most important forms of protein damage/loss, relevant to both medicine and food science. Named after the pioneer in the field, the Maillard reactions were described in 1912[1] and systematically presented for the first time by John E. Hodge in 1955[2]. During the early years, glycation was studied in the context of food science, food processing and hence relative to health via nutritional intake. In 1977, a fraction of Hb, HbA1c, was identified as a ketoamine (glycation product) and the concept of *in vivo* protein glycation gradually became mainstream[3–5]. HbA1c was proposed as a useful biomarker for diabetes monitoring[3,4], and endogenously produced advanced glycation endproducts (AGE) have since attracted further scientific attention, beyond food chemistry, from fields including medical biochemistry and pathology.

Importance of glycation for health

Glycation and the AGE–RAGE axis

The study of the role played by glycation in disease pathogenesis originally relied on measuring fructosamine levels in biological fluids, combined with the

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characterisation of endogenous AGE in the circulation and tissues[6,7]. These measurements were related to glycaemia and the topic very much focused on diabetes[3–5].

In hyperglycaemia (post-prandially or in non-controlled diabetes) and to a lesser extent in normoglycaemia, both circulatory proteins and proteins of the endothelium are exposed to (excess) glucose, leading to the slow formation of AGE[8–11]. During that process, glycation adducts are created on the protein molecule, as a function of glucose levels. Accumulation of glycation adducts on the protein promotes excessive cross-linking with other protein molecules, which, in the case of collagen for example, would inhibit the formation of an ordered and functional polymeric complex. Such changes could lead to the formation of a thick vascular wall with (1) reduced elasticity and (2) a high affinity of collagen to bind other circulating proteins such as IgG, albumin and lipoproteins such as LDL[12–21]. In turn, the immobilisation of proteins on the vascular wall will promote further glycation and cross-linking and will act as a signal for chemoattraction of macrophages and monocytes, promoting inflammation and 'foam' cell formation in the endothelium[22–24].

The discovery that AGE can bind on cellular receptors and alter intracellular events was a breakthrough, linking glycation to signalling[25]. Receptors such as AGE-R1, AGE-R2, AGE-R3, MSRII, CD36, LOX-1 and the receptor for AGE (RAGE), the most characterised receptor[26], are multi-ligand cell-surface Ig, with the ability to initiate injury-like intracellular events, mainly expression of genes related with inflammation and oxidative stress[27–29]. Upon activation of RAGE, intracellular reactive oxygen species levels are increased through up-regulation of NAD(P)H oxidase expression. This in turns leads to activation of the Ras-mitogen activated protein kinase pathway, ultimately up-regulating NF-κB and the production of inflammatory molecules (including TNF-α, vascular cell adhesion molecule 1, intercellular adhesion molecule 1 and IL-1β). The up-regulation of NF-κB also initiates a positive feedback loop that sensitises the cell (and hence the tissue) to AGE by promoting RAGE production[24].

Together accumulation of AGE in tissues and AGE–RAGE interactions are the two main pathways of glycation involvement in disease pathogenesis. These two pathways are often acting simultaneously and their individual effects are hard to distinguish; hence they are commonly presented in the same context when discussing glycation-related pathophysiology[12,30–34].

Glycation and health throughout the lifecycle

Glycation is relevant to all stages in the lifecycle, including conception and early gestation. The reproductive tract is a known site for AGE accumulation both in men[35] and women[36]. AGE accumulation is followed by changes in the distribution of RAGE in reproductive tissues[37], and the soluble isoform of RAGE in seminal/follicular fluid[38,39], which may lead to lower sperm quality[38], lower likelihood of success following assisted reproduction[40,41] and reduced embryonal quality and development[39,41,42]. During the course of pregnancy, activation of the AGE–RAGE axis may be involved in the pathogenesis of preeclampsia[43–45]. So far, evidence on the involvement of AGE and/or RAGE in fetal development is limited and based on animal studies. For example, a study on transgenic mice showed that overexpression of RAGE was associated with impairments in alveolar morphogenesis. The degree of RAGE overexpression was related to the magnitude of the abnormality with homozygous mice having histological changes similar to human bronchopulmonary dysplasia. The study also found that these early life changes could lead to increased risk of 'destructive' emphysema[46]. Glycation has also been proposed as a mechanism of ageing[47,48]. Evidence from animal models suggest that a diet low in AGE (50 % reduction in AGE intake) was associated with amelioration of insulin resistance, lower AGE accumulation (both indications of the ageing process) and ultimately increased lifespan compared with the controls[49]. Similarly, mice on caloric restriction, a popular model of lifespan expansion in animal models, have lower levels of collagen cross-linking and lower levels of lens cataract, suggesting lower AGE accumulation in the vitreous and the extracellular matrix[50,51] as well as in the brain[52]. In fact, mice fed high AGE diets while on caloric restriction did not show any increase in their lifespan and the authors of the report suggested that lower AGE intake may be one of the mechanisms behind the caloric restriction model[49,53]. An interesting observation linking the effect of AGE in ageing and as early in life as in conception comes from a study showing the active involvement of AGE accumulation in ovarian ageing and ovarian function in human subjects[54].

HbA1c and risk of chronic diseases

Even though the exact mechanisms of disease pathogenesis remain elusive, extensive evidence is available to associate glycation with disease risk. Glycation has a particular relevance for age-related diseases, including Alzheimer's disease[55,56], skin ageing[48] and cataract[47]. These conditions are characterised by increased, possibly lifelong, deposition of AGE in the affected tissue[57–59].

As *in vivo* glycation is believed to be mainly driven by plasma glucose concentrations, the most established relationship is between glycation and diabetes. HbA1c is the gold standard method for diabetes diagnosis and monitoring[60]. According to the American Diabetes Association, individuals with HbA1c levels between 5·7 and 6·5 % are considered at high risk of developing diabetes. Those with HbA1c > 6·5 % are classified as having diabetes[61]. Among patients with diabetes, higher HbA1c levels are associated with increased risk of retinopathy[62–66], neuropathy[67] and nephropathy[66].

Glycation has recently attracted attention as a risk factor for normoglycaemic individuals. For the purpose of the present paper, we conducted a systematic literature search to identify studies documenting the effect of increased glycation on the risk of non-communicable chronic diseases in normoglycaemic subjects. We identified fifteen reports from eight studies (European Prospective

Investigation into Cancer and Nutrition[68,69], Athero-sclerosis Risk in Communities study[70–73], Australian Diabetes, Obesity and Lifestyle study[74], the Hoorn Study[75,76], Framingham Offspring[77], Rancho Bernardo[78], Women's Health Study[79–81] and National Survey of Cardiovascular Disorders 1990[82]) analysing data from a total of over 63 000 participants, followed-up for 4–15 years. The outcomes of interest were diabetes risk, CVD, IHD, stroke, CHD and all-cause and CVD mortality. Two reports focused on the association between glycation and cancer risk, especially colorectal[69] and breast cancer[80]. Overall, the studies showed a positive relationship between higher HbA1c and the risk of stroke and/or CVD and/or mortality ranging between 18 and 55 % higher risks per 1 % increase in HbA1c[68–74,82]. As far as cancer incidence is concerned, the results are still inconclusive. Data from the European Prospective Investigation into Cancer and Nutrition cohort suggest a 33 % increase in the incidence of colorectal cancer per every 1 % increase in HbA1c[69], but an analysis of the Women's Health Study data did not find any association between HbA1c and breast cancer risk[80]. As the two cancer types differ significantly in aetiology, colorectal cancer has a strong dietary link[83] whereas breast cancer is mainly of genetic aetiology[84]; more research is needed before any conclusion is reached.

Oxidative stress and protein glycation in normoglycaemia

As observed by Selvin et al.[70], fasting glucose may fail to explain the positive relationship between HbA1c and CVD and/or mortality. Correction for classical risk factors (including smoking, dyslipidaemia and inflammation) explain the relationship better[75,76,79,81], suggesting that a shared mechanism may drive the increase in HbA1c levels. Although indications and potential mechanisms are in place to suggest an active involvement of oxidative stress in protein glycation in normoglycaemia and hence the increase in the risk of chronic diseases, so far little evidence is available to support such a hypothesis.

In our previous work, we hypothesised that oxidative stress could be this shared mechanism, which acts as a glycation driver in normoglycaemia.

Using the Scottish Health Surveys datasets 1993–2010, we have shown that, in individuals without diabetes and HbA1c levels lower than 6·5 %, age–sex adjusted HbA1c levels are positively correlated with smoking status, an association seen even among ex-smokers who used to smoke regularly[85]. Smoking status was used as a proxy for oxidative stress and, in a similar way, fruit and vegetable intake was used as a proxy for antioxidant intake. Smoking was positively associated with HbA1c levels from as few as ten cigarettes per day a finding consistent with previous reports[86,87] (Fig. 1). The likelihood of having an HbA1c level within the prediabetes range (5·7–6·4 %) was double among smokers compared with non-smokers; this was seen even with less than ten cigarettes per day smoked. Interestingly, smoking cessation does not lead to complete reversal to the non-smoking
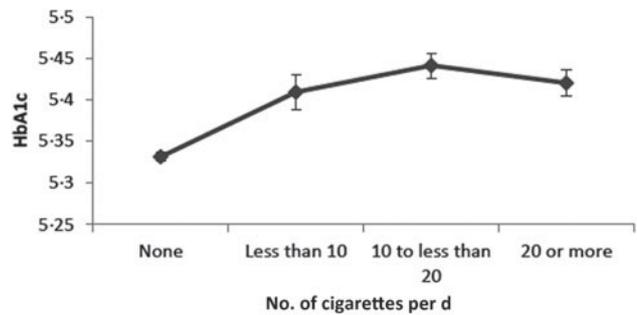

Fig. 1. Age–sex adjusted mean (SD) of %HbA1c according to number of cigarettes per day (adapted from Vlassopoulos et al.[85]).

state, as former smokers were found to have lower HbA1c levels than smokers but not as low as never smokers[86,87]. In a linear regression model, smoking was associated with 0·08 % higher HbA1c compared with no smoking, which is equal to 0·25 times the SD. As expected, vegetable intake had the opposite effect being associated with lower age–sex adjusted HbA1c levels with more portions consumed. In fact, for every extra 80 g portion of vegetable consumed there was an associated 0·01 % reduction in HbA1c.

The hypothesis that glycative and oxidative damage are closely related *in vivo* is supported by evidence showing that in purified plasma albumin, oxidative damage, measured as a reduction in free thiol groups, was positively related to glycative damage, measured as fructosamine and carbonyl rate[88]. Moreover, Cys-34, a key site of oxidative damage in albumin *in vivo*[89], has also been suggested as a glycation site, especially from α-oxoaldehydes[90]. Since *in vitro* models are often removed from physiologically relevant reactions, it is important to set up mechanistic studies with adequate parameters. To test the hypothesis that, in normoglycaemia, oxidative stress promotes glycation, we carried out 4-week albumin incubation studies (albumin has a half-life of 14–28 d). Glucose concentrations 5 and 10 mM were employed to replicate normoglycaemia and (non-controlled) diabetes, respectively, whereas 20 and 30 mM glucose were used as the positive controls (supraphysiological concentrations). There is no consensus on the plasma levels of H_2O_2 (from nearly 0 to 35 μM[91–93]); we used a low concentration H_2O_2 (10 nM) to simulate physiologically relevant oxidative stress[94]. Co-incubation of albumin with glucose and physiological levels of H_2O_2 led to significantly higher glycation at all glucose levels tested, after 2 and 4 weeks incubation, compared to glucose alone. At the physiological glucose level (5 mM), there was no significant glycation (v. negative control) in absence of H_2O_2 (Fig. 2), indicating that oxidative stress plays an important role in glycation in normoglycaemia. Physiologically, in the presence of oxidative stress, proteins can get quickly oxidised and remain in this form in circulation until they are degraded by proteases[95]. As extracellular/circulating proteins are more likely to get oxidised first before getting glycated, due to the relative speed of the reactions, the same experiments were repeated using pre-oxidised protein. The pre-oxidised bovine serum albumin led to a higher production of fructosamine
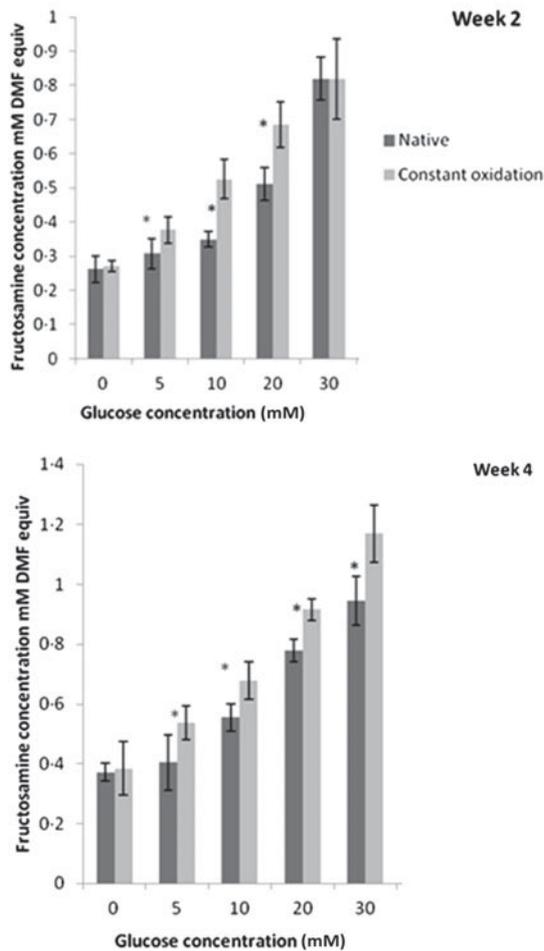
Fig. 2. Differences in fructosamine concentration after incubation with glucose alone compared to glucose and constant exposure to oxidation from hydrogen peroxide (10 nм) after 2 and 4 weeks incubation. *$P < 0.05$ native *v.* constant oxidation; fructosamine was measured using the nitroblue tetrazolium method with the synthetic fructosamine equivalent deoxy-morpholino-fructose (DMF) as a calibrator (adapted from Vlassopoulos *et al.*[96]).

when incubated with glucose as compared with the native incubated BSA. Oxidative stress also drove glycation of human plasma proteins, in presence of 5 mм glucose.

Brought together, these results[85,96] indicate the potential role for oxidative stress as a driver for glycation in normoglycaemic individuals. The increased levels of HbA1c seen in smokers and those consuming low amounts of fruit and vegetables could be partially due to their impaired redox status, as stipulated by the epidemiological data. This interaction between oxidative stress and glycation will be subtle but with potentially sizeable long-term effects. Hence, dietary interventions aiming to restore the antioxidant/pro-oxidant balance in subjects at high risk of oxidative stress could be of value in chronic disease prevention.

Antiglycative capacity of antioxidants and polyphenols

In the search for compounds able to inhibit or slow the glycation reaction, antioxidants have attracted attention.

The first AGE blocker identified is aminoguanidine[97]; a dicarbonyl scavenging agent that reduces AGE production by removing the oxidatively produced precursors, like α-oxoaldehydes[98,99]. Aminoguanidine, like other glycation-inhibiting compounds aspirin and ibuprofen, has the capacity to scavenge free radicals and improve redox status, which may contribute to their antiglycative capacity[99–101].

The antiglycative capacity of antioxidant vitamins and polyphenols has also been investigated, with *in vitro* studies showing some polyphenols and phenolic acids to be even more effective than aminoguanidine in inhibiting glycation[102–104]. Herb extracts and commonly consumed herbal preparations have been shown to inhibit glycation of albumin in experimental settings. Red wine, green tea, maté tea (Ilex paraguariensis)[105,106], cinnamon, garlic[107] and other herbs used to prepare hot drinks or added during cooking are rich in a variety of micronutrients with antiglycative effects[108,109]. A recent review of the literature by Xie *et al.*[110] analysed results from nineteen *in vitro* trials and eleven animal studies and concluded that antiglycative capacity of polyphenols is linked to ring hydroxylation patterns. In this context, molecules with hydroxyl groups in the A and B rings (i.e. apigenin < luteolin, fisetin < quercetin, daidzein < genistein) those with multiple hydroxyl groups especially in the *ortho*- and *meta*-structure (i.e. phloridzin < sieboldin), the proanthocyanidin di/trimmers and the ellagitannins all showed increased antiglycative capacity. On the other hand, hydrogenation of the C_2–C_3 bond (i.e. eriodictyol < luteolin), methylation (i.e. diosmetin < luteolin) and the addition of rutinosides all decreased the antiglycative capacity[110]. The results of *in vitro* studies are still heterogeneous and a thorough review of the glycation models and assays used would help to understand why translation of the findings to a physiological setting has not been forthcoming. Some of the reasons include use of high glucose or fructose concentrations, supraphysiological concentrations of polyphenols/phenolic acids, use of compounds with very limited bioavailability, and variability in the incubation period/temperature. Doses tested *in vitro* are, mostly beyond concentration that could be reached via habitual consumption of phenolic-rich foodstuff. Most polyphenols are metabolised extensively in the gut and by the liver after ingestion, and have generally a low bioavailability[111,112]. Therefore studies focusing on the systemic effects of the 'parent' compounds, as found in foods, are likely to have low translational values. Phenolic acids, such as 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and caffeic acid, conversely, are formed after exposure to the gut microbiota, have a higher bioavailability than larger polyphenols and are more likely to exert systemic effects[111,112].

Despite the extensive mechanistic evidence, epidemiological data on polyphenol consumption are scarce. Principal reasons include the difficulties and the biases associated with deriving polyphenol intake data from dietary records. The process involves the use of databases, such as PhenolExplorer[113] documenting the polyphenol content of foods[113,114] and/or the analysis of

FFQ to identify patterns of higher intake of polyphenol-rich foods. So far, there are no reports addressing the relationship between polyphenol intake and glycation levels. The reports associating polyphenol intake with diabetes risk have so far reached contradictory conclusions[115–117]. Our own systematic review of the literature relating antioxidant intake with protein glycation in normoglycaemia showed that human trials with polyphenol-rich supplements and foods are few and characterised by high heterogeneity, poor design and small samples size (in preparation). In the last 20 years, only fourteen trials used polyphenols as a means to reduce glycation in non-diabetic individuals, out of which two did not have any control group[118,119]. Taken together, the results of these studies seem to suggest that polyphenol supplementation fails to improve glycation markers in non-diabetic individuals, although this conclusion is most likely to be a result of poor study design. In populations with established impaired glucose tolerance, increased intake of polyphenols might be promising in reducing protein glycation[120,121], but no hard conclusions can be made at this point. The bioactive molecules tested were diverse with no standardisation in dose. The majority of the studies had glycation as a secondary outcome, leading to low statistical power, and did not have sufficient duration to detect changes, if any were present.

Considerations for the future

Although the importance of glycation as a marker of disease pathogenesis outside of diabetes is becoming clearer, it is yet to be fully understood. More studies are required to describe the interactions between oxidative stress and glycation, especially in normoglycaemia. The importance of RAGE activation to signal intracellular events that promote dysfunction and the factors that determine the levels of the soluble isoform of RAGE have not attracted the required attention.

As far as polyphenol and antioxidant trials are concerned, there is still much improvement to be done in terms of study design before conclusions can be reached. If the working hypothesis is that polyphenols will exert health benefits via their antioxidant capacity, then markers to document such improvements should be included and results on glycation markers, such as HbA1c, should be discussed alongside oxidative stress improvements.

Sample size and targeting the correct population are two key aspects of the study design to be considered. Polyphenol supplementation in a relatively healthy population is likely to have a subtle effect on health markers and hence studies with large sample sizes are likely to be required[122]. The majority of the studies to date fall short of that sample size and are hence likely to be underpowered. As a result, we should be careful in concluding that polyphenol supplementation has no effect on glycation. The current literature may be just describing a lack of power to detect such an effect if any.

A good understanding of the supplement used, with data on bioavailability, composition and dose would allow for a more effective comparison of the studies.

Also ensuring that the study duration is sufficient to detect changes in glycation markers is a vital improvement. Albumin has a half-life of 14–28 d whereas Hb half-life is 90 d; studies with duration shorter than the half-life of the target protein are unlikely to detect any changes in protein glycation. Also even though physical protein damage is the main pathway of glycation-related pathogenesis; RAGE activation, the soluble isoform of RAGE levels and glycation-related inflammation are also important pathways for the involvement of glycation in disease pathogenesis, but are so far understudied[123,124].

Conclusion

Glycation is an important mechanism of end organ damage and disease pathogenesis affecting individuals throughout the lifecourse. With many target molecules and mechanisms of actions glycation and oxidative stress are increasingly recognised as of clinical importance not only in diabetes but in normoglycaemia as well. Epidemiological and *in vitro* data so far are supporting the hypothesis that oxidative stress and its regulation with antioxidants is of importance in an attempt to inhibit glycation, especially in normoglycaemia. Although the importance of nutrition in glycation regulation is becoming more apparent, clinical trials with polyphenols so far lack the quality to form conclusive decisions. More large-scale and high-quality interventions are needed before recommendations can be made.

Conflicts of Interest

None.

Authorship

A. V. conducted the studies described and drafted the manuscript. E. C. and M. E. J. L. secured the funding, supervised the studies and contributed to the drafting of the manuscript.

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