Association between advanced glycation end products and intake of different types of beverages in a healthy population of students

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MAJA VICTORIN

ASSOCIATION BETWEEN ADVANCED GLYCATION END PRODUCTS AND INTAKE OF DIFFERENT TYPES OF BEVERAGES IN A HEALTHY POPULATION OF STUDENTS

Diploma Thesis

Academic year:
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Mentor:
Associate Prof. Ivana Kolčić, MD, PhD

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Abbreviations

AGEs, advanced glycation end products

CKD, chronic kidney disease

CML, Nε-carboxymethyl lysine

CVD, cardiovascular disease

FFQ, food frequency questionnaire

HFCS, high fructose corn syrup

NF-kB, nuclear transcription factor kB

RAGE, receptor for advanced glycation end products

SAF, skin autofluorescence

SIF, skin intrinsic fluorescence

WHO, World Health Organization

SSB, sugar-sweetened beverage

1. Introduction

An increase in skin autofluorescence (SAF) has been found to be associated with the development of cardiovascular disease, diabetes mellitus type 2 and chronic kidney disease, among many other diseases. Measurement of skin autofluorescence can be performed with an AGE reader, a machine aimed at checking the amount of advanced glycation end-products (AGEs) in a noninvasive manner.

1.1 Definition

Advanced glycation end-products (AGEs) represent an emerging biomarker for the monitoring and measurement of the development of peripheral arterial disease, cardiovascular disease, diabetes mellitus and chronic renal failure. AGEs are produced by non-enzymatic glycation of proteins and lipids or during oxidative reactions (1). During oxidative stress AGEs are formed as a reducing sugar chemically modifies a protein by adding an element. The addition of a ketone or aldehyde group creates a stable structure that can combine, cross-link and accumulate with proteins, and later on lead to a slow turnover and a buildup of harmful products (2)

1.2 Physiology of AGEs

AGEs are part of the normal metabolism in our body and only an excess of these products will have a negative impact on the organism (3). The pathological effects are mainly seen during the time of oxidative stress, which is also promoted by AGEs (4). Oxidation happens during a state of metabolic stress in the organism; when free radicals outweigh the number of antioxidants able to scavenge these products. In the event of stress, the reactive oxygen species travel freely and cause permanent damage to the body on a cellular level. Upon binding to the cellular receptors for AGEs (RAGE) the accumulation of cross-linked larger structures leads to interference of the collagen production and down the line cause vessel stiffness of interstitial tissue and mitochondrial dysfunction (5). Binding of the AGEs to their receptor causes the activation of intracellular signaling pathways of pro-inflammatory genes and eventually propels the atherosclerotic process forward and has detrimental effects on our health (6). Figure 1 shows the different pathways of formation of AGEs, via the production of reactive dicarbonyls. Maillard reaction, glucose autoxidation, lipid peroxidation, glycolysis and polyol pathway all lead to the formation of dicarbonyls, which occur during food processing, storage and physiological conditions (7, 8).

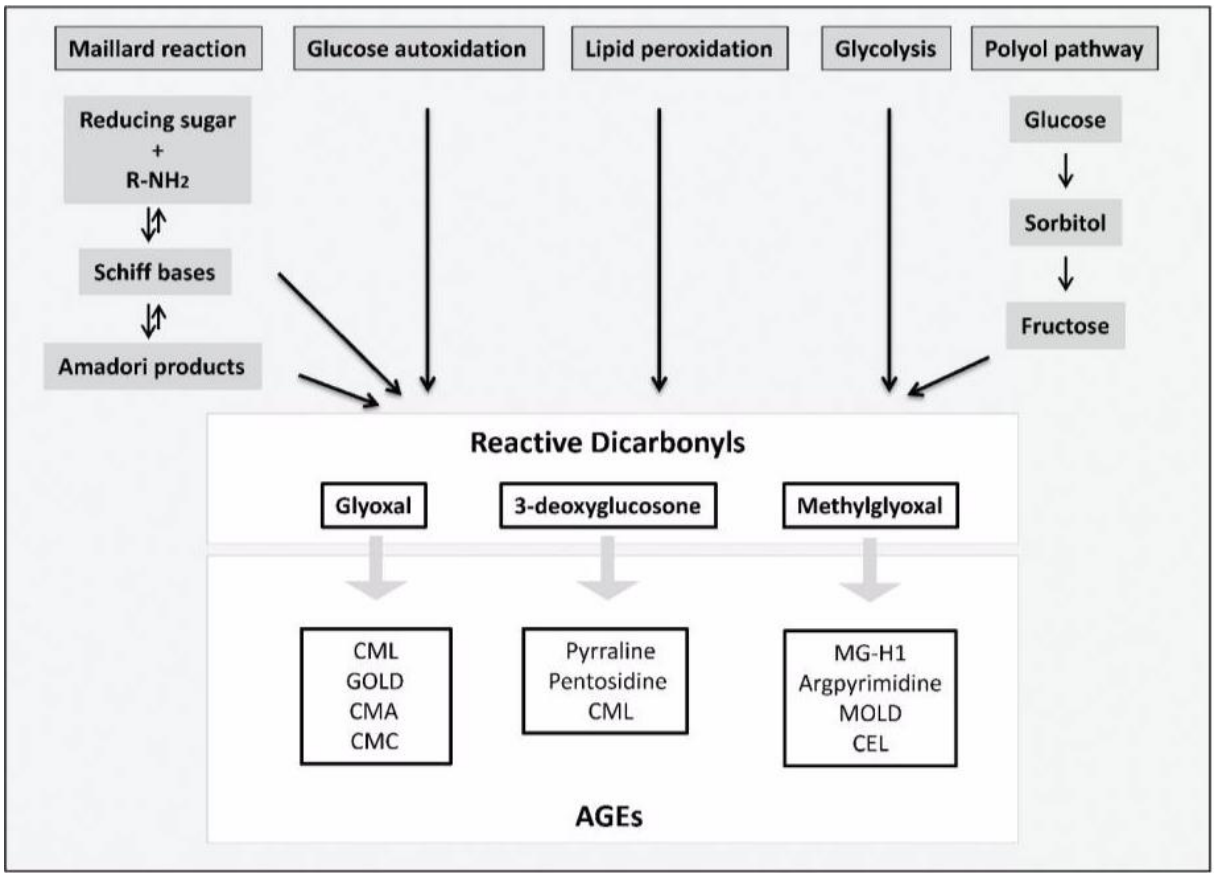

Figure 1. Physiology of the formation of AGEs and the possible pathways in vivo (Available from: 7, 8).

An elevation of free radicals can be seen in individuals who smoke, have a high intake of processed vegetable oils, meat, sugar-sweetened beverages, energy drinks and alcohol (8, 9). As well as in patients with chronic diseases and a state of long-term psychological distress (10). The long-term deleterious effects of the free radicals in the human body are consequences of damages in the DNA, which include base modifications and strand breaks. Similar DNA damage can be seen following ionizing radiation exposure (11).

In a normal healthy individual the reactive oxygen species are not able to carry out the disastrous effects due to built-in coping mechanisms, such as DNA-repair and antioxidants that bind the reactive oxygen species.

Advanced glycation end-products (AGEs) have a negative impact on nearly any type of cell and are specifically thought to be involved in the process of aging as well as in the development of several chronic diseases, such as diabetes mellitus, atherosclerosis, cardiovascular disease and chronic kidney disease. The development of AGEs is more likely to occur when blood sugar is elevated, which explains the higher levels of AGEs in people with

poorly controlled type 2 diabetes (2, 11). Cigarette smoke has been found to be an exogenous source of AGEs and has a significant effect on skin autofluorescence (SAF) (12).

1.2.1 AGE receptor

The action of AGEs is activated through cross-linking and by binding to the multiligand cell-surface receptor – receptor for AGE (RAGE). Upon binding to the RAGE, an array of signaling cascades are activated and consequently lead to changes in gene expression and it eventually causes an increase of proinflammatory markers and oxidative species, further aggravating the oxidative stress in the body and leading to irreversible damage (13). RAGEs are expressed in a wide variety of tissues, including the vasculature, lung, heart, and endothelium, as well as in many other cells (14).

Figure 2 presents the pathophysiology of how AGEs produce arterial stiffness and endothelial dysfunction by binding to the receptor for AGEs in vascular tissues. Upon formation of AGEs in the setting of free radicals, nuclear transcription factor kB (NF-kB) is up-regulated (15). Activation of NF-kB triggers inflammation and formation of reactive oxygen species and eventually leads to arterial stiffness (14).

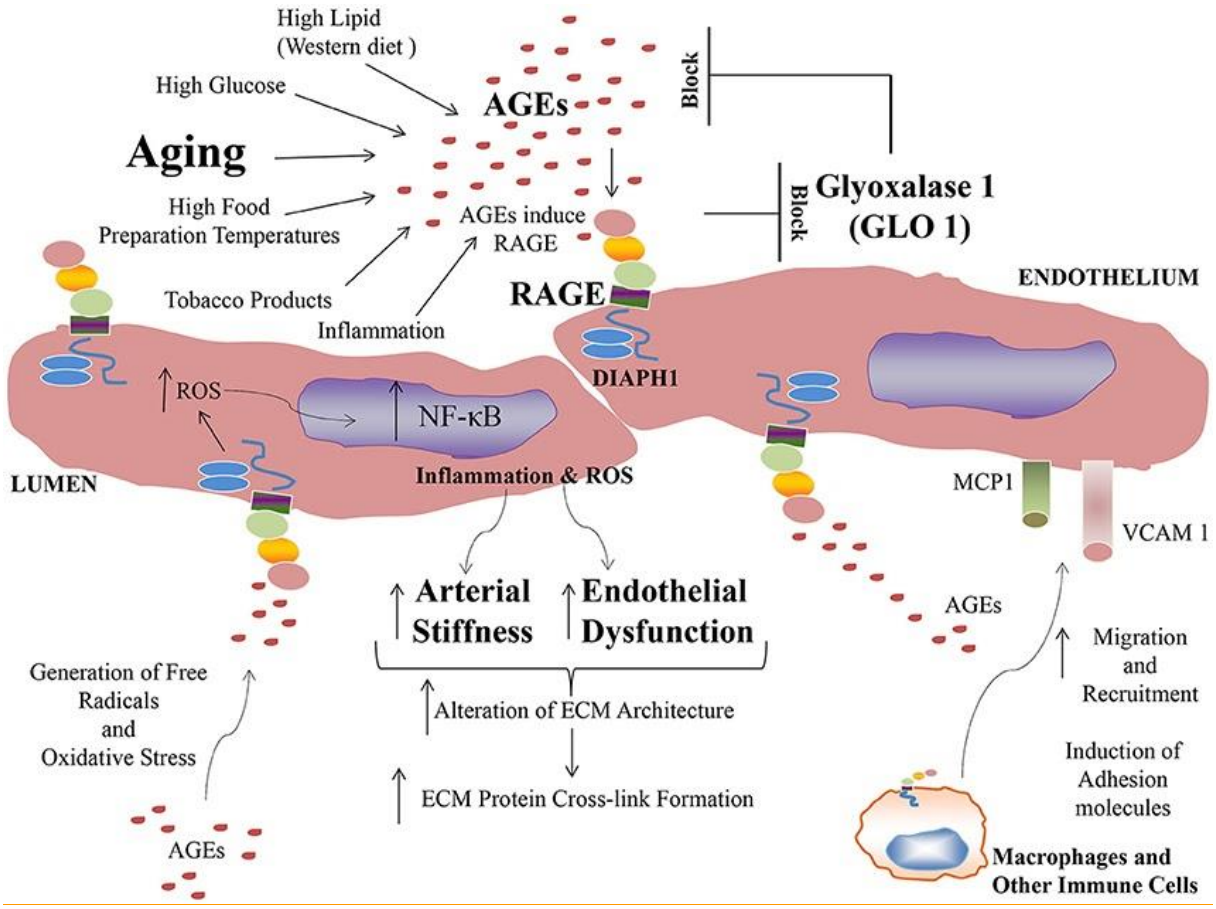

Figure 2. The pathophysiological pathways of AGEs in vivo (Available from: 14).

1.3 Measurement AGEs

AGEs can be found in many different body compartments and in a variety of different shapes, bound or unbound to proteins. Due to the broad variation of AGEs in tissues, no standardized method to measure these products has been accepted as an official method. There are a few approaches used to obtain a value of AGEs, one of them being based on blood samples. However, since AGEs are thought to be formed mainly intracellularly, the concentration in the blood is not reliable. Due to the fluctuation and short half-life in the blood, these AGEs do not correlate to the amount of AGEs in other tissues (16, 17). Other sites with AGEs of longer half-life can be found in the eye lens, cartilage and the skin (18). Traditionally, AGEs were quantified using enzyme-linked immunosorbent assay (ELISA), and ultra-performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS) (19). High liquid chromatography with ultraviolet or fluorescence detection (HPLC-UV/FD) is another option (20). Gas chromatography with mass spectrometry (GC-MS) is used to analyze

AGEs in food (21). And a company from the Netherlands, called DiagnOptics, use intrinsic skin autofluorescence to develop a non-invasive method (2).

To measure the amount of AGEs in food products researchers have found that Nε-carboxymethyl lysine (CML), a main component of advanced glycation end products, correlates with the level of AGEs in a certain food type and can be derived from protein and lipid glycoxidation (22). Since CML is a stable product it has been proposed for the use as an indicator of the nutritional value of food that has undergone the process of heating through cooking (23).

1.3.1 AGE reader

The AGE Mu Reader device (DiagnOptics, Groningen, Netherlands) is a non-invasive monitoring device that emits ultraviolet light to excite autofluorescence in the skin of humans. The autofluorescence is created from the build-up of Advanced Glycation End-products (AGEs). In only 12 seconds the device provides a risk assessment and helps in determining the status of chronic diseases involving the cardiovascular and renal systems (24).

The volar side of the forearm is placed over the window of the AGE reader. Ultraviolet light is emitted from the machine and AGEs molecules of the skin become excited, and in turn emit fluorescent light that can be detected by the reader. The wavelengths correlate with the quantity of AGEs in the skin (25).

1.3.2 Interpretations of the AGE reader measurements

The AGE reader measurements consist of an AGE score and a scale comparing the results with chronological age. An increase of AGEs in the skin correlates with an increased risk for both type 1 and 2 diabetes mellitus and cardiovascular disorders (26). Skin autofluorescence (SAF) has been shown to be significantly associated with age, coffee intake, smoking, high blood pressure, HbA1c and diabetes (27, 28).

The skin fluorescence measurements may be influenced by dark pigmentation, certain skin diseases masking the healthy skin at the site of measurement or the usage of skin products, fasting state, and lifestyle factors (29).

Skin autofluorescence (SAF) levels may not be reliable in darker skin types (Fitzpatrick class V-VI) and several skin products may affect the measurement of SAF, especially sunscreens and skin tanners (29). When possible, the subject being tested should be asked to avoid the usage of skin products a few days before performing a SAF measurement.

Findings of the AGE reader is aimed at discovering and identifying individuals at an increased risk of metabolic syndrome, diabetes mellitus and cardiovascular disease.

1.4 Effect of accumulation of AGEs

1.4.1 Diabetes mellitus

Diabetes mellitus (DM) is a group of chronic, metabolic disorders characterized by hyperglycemia, which over time advance to long term deleterious effects on both micro- and macrovasculature, leading to systemic damage of the heart, kidney, eyes and nerves. The most common type is diabetes mellitus type 2, which develops when resistance to insulin develops or the pancreas cannot produce enough insulin. Previously it was more prominent in the adult population, however with obesity being on the rise among the younger ages due to poorer diet, it is now prevalent in this population as well (30).

Type 1 diabetes is an autoimmune disease in which the beta cells of the pancreas are affected, and the production of insulin becomes insufficient.

As presented in Figure 3, one of the most severe and common long-term side effects of diabetes is atherosclerosis and the development of cardiovascular disease, which in turn can lead to stroke and other cardiovascular outcomes. These deleterious effects are particularly pronounced in a setting where AGEs are increased (31). Factors that play a key role in the formation of AGEs include the rate of protein turn over, the presence of oxidative stress in the environment and the degree of hyperglycemia, particularly present in uncontrolled diabetes (9, 32). The increased level and accumulation of AGEs are among the leading causes of long-term detrimental effects of DM on micro and macro vasculature (33, 34).

The World Health Organization (WHO) states that 422 million people around the world are affected with diabetes mellitus type 2, causing a concern for the load on health-care system (35). Being able to test and measure the diabetic status in the population is of utmost importance and the development of a reliable predictor of cardiovascular outcomes is essential in addition to the already well established HbA1c. HbA1c refers to glycated hemoglobin (A1c), which is produced as the body processes sugar and glucose in the bloodstream attaches to hemoglobin. The formation of sugar-Hb linkage indicates the presence of excessive sugar in the bloodstream, when values are above 6.5%, and is primarily used to determine the average blood sugar level from the previous three months, as an assessment test for glycemic control in diabetic patients (9, 30). The test is limited to three months since the average lifespan of a red blood cell is 120 days or four months.

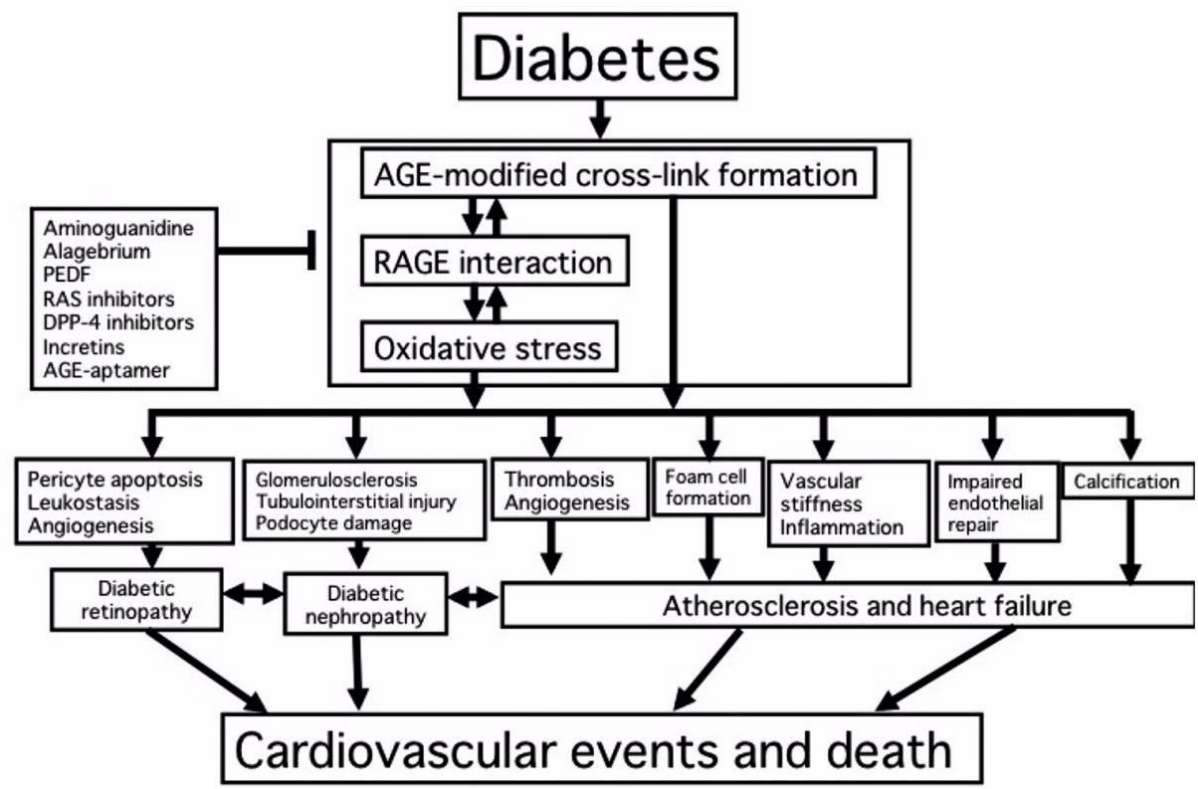

Figure 3. The pathophysiological mechanism of AGEs in diabetic vascular complications (Available from: 36).

1.4.2 Cardiovascular disease

Cardiovascular disease (CVD) is a general term for conditions affecting the heart or blood vessels. It's usually associated with a build-up of atherosclerosis of the arteries and comes with an increased risk of plaque formation. The development of CVD can be linked to both epigenetic factors and lifestyle choices, especially diet and lack of exercise.

AGEs are also one of the culprits in the development of cardiac dysfunction through two major mechanisms: cross-linking of proteins, leading to stiffening of vessel walls, and via the binding of AGEs to their cell surface receptor (RAGE) (31, 36). Through these pathways' plaque formation is accelerated, as well as increased cardiac fibrosis, which eventually leads to a decrease in cardiac function. The physiological process of cross-linking of collagen is of importance when it comes to tissue structure, as long as flexibility is not compromised. The accumulation of AGEs alters the normal function and flexibility so that the turn-over of proteins are decreased and thus leading to bonds highly resistant to degradation (32). The cross-linking of myocardial and vascular collagen affects the innate properties of these structures and will

eventually result in decreased vascular elasticity and myocardial flexibility, as well as an increased myocardial stiffness (37).

1.4.3 Chronic kidney disease

The renal function is an indicator of the health status of the kidneys and is measured through the glomerular filtration rate (GFR), which describes the flow rate through the kidney. The normal glomerular filtration rate of a healthy person ranges from 90 to 120 mL/min/1.73 m^2 (38).

Chronic kidney disease is the process of decline of renal function over time and the most important risk factors for its development are hypertension and diabetes mellitus. It is measured via an estimated glomerular filtration rate (eGFR) and can be divided into different stages depending on severity and advancement: G1 >90, G2 60-90, G3a 45-59, G3b 30-44, G4 15-29, G5 <15 mL/min/1.73 m^2 (39).

The majority of the AGEs are excreted and eliminated through the kidneys (40). When passing through the tubular system AGEs are broken down by the proximal renal tubular cells and the ones that remain are excreted in the urine.

In the setting of oxidative stress, the function of the renal cells is decreased and consequently it will lead to renal impairment characterized by glomerular filtration damage and subsequent reduction in AGEs clearance (36). Which later may promote renal injury by further increasing body tissue and circulating AGEs pool (29, 37).

In a hyperglycemic state the kidneys are affected by the high levels of AGEs due to an up regulation of the receptors for these molecules, RAGE, particularly high in concentration in the podocytes found in the Bowman's capsule of the glomerulus (41). As the RAGEs are activated, stimulation of the expression of Vascular Endothelial Growth Factor (VEGF) is increased in its expression and activity, leading to hyperpermeability of the glomerulus and attraction of inflammatory markers, particularly Tumor Growth Factor beta, TGF-β (42). TGF-β has a deleterious effect on the renal tissue by acting on mesangial cells, podocytes, endothelial and tubular cells. Through an increase of extracellular matrix production, a hypertrophy of the basement membrane is produced, dysfunction of apoptosis and podocytes leads to a decrease in the function of the glomerular filtration rate and tubular dysfunction, and in turn a permanent renal deterioration (43, 44).

1.5 AGEs in food and drinks

1.5.1 AGEs in food

In conjunction with the AGEs that form in our bodies, these molecules also originate from the food that we eat and drinks that we consume. An especially high content of AGEs can be seen in foods that have been exposed to high temperatures and dry heat, cooked meat, deep-fried processed food, fried eggs, dairy products and margarine (45). The process of heating of food will cause an increase of AGEs by 10-100-fold when compared to the raw product (32). For example, an egg cooked through poaching or medium heat in a pan has fewer AGEs than if cooked on high heat in a frying pan with butter. Kellow *et al*. recently showed that dietary intake of meat and meat products was positively associated with SAF in a sample of 251 healthy adults (28). The process of browning and flavor development of food, through cooking, results from the interaction between glucose or reducing sugars and proteins leading to the formation of a Shiff base and eventually an Amadori product, which is called a 'Maillard reaction', as demonstrated in Figure 4. Slow changes made to the Amadori products will eventually lead to the formation of AGEs (45, 46). The ingestion of food cooked in this manner causes a direct ingestion of AGEs (47).

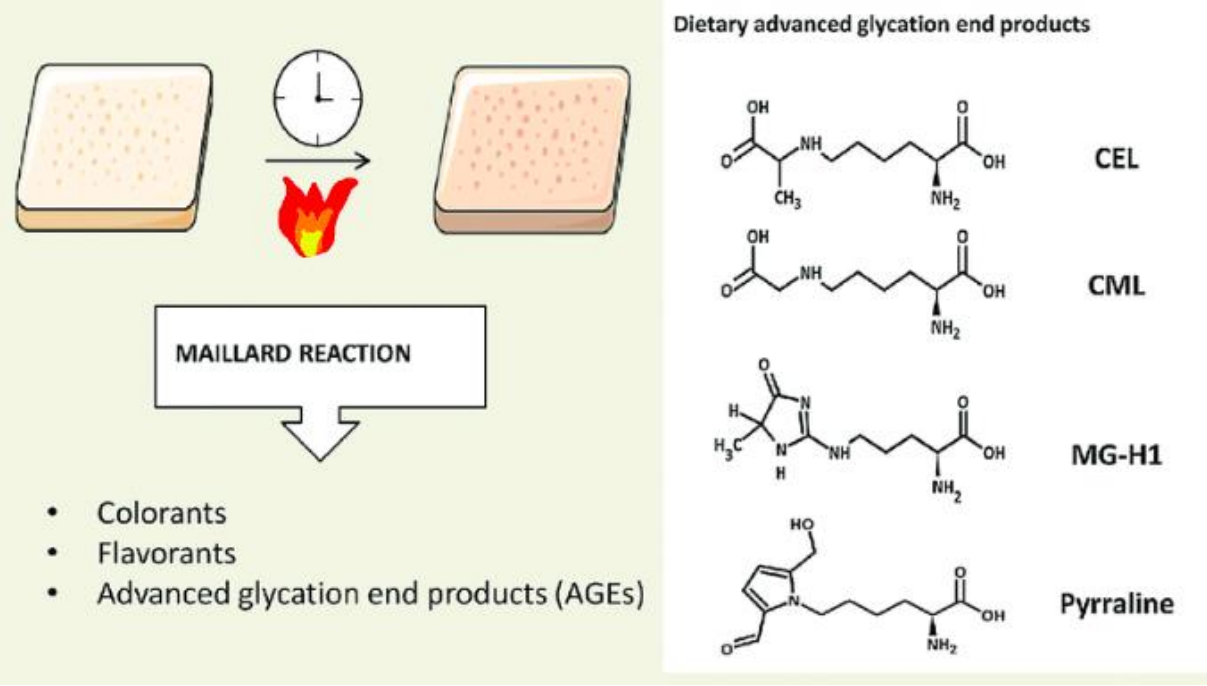

Figure 4. Presenting the Maillard reaction which eventually leads to the coloring of food, flavor enhancement and AGE production, due to cooking over high heat (Available from: 8, 45).

Foods particularly low in AGEs are fruits, nuts, vegetables, legumes and whole grains, especially if prepared on low heat through steaming, boiling and limited use of fat. They can even reduce the buildup of AGEs that have occurred during time (10).

One of the most well-known diets around the world is the Mediterranean diet, being known for its health benefits due to the high amount of fruit and vegetable intake, the usage of olive oils, fish, tea, coffee and red wine (48, 49). Adherence to a Mediterranean diet creates an overall positive effect due to its low content of AGEs and other metabolites that triggers oxidative stress and AGEs formation (50, 51). A study also found that it might have protective effects against chronic diseases *(*52). The consumption of red wine in this population has been shown to be low to moderate, without excess (53).

Studies have shown that adding acidity, such as lemon juice or vinegar, to meat before cooking by marinating for ten minutes, significantly lowers the value of AGEs, as well as shorter cooking time and cooking at lower temperatures (11). Other studies found that overweight women who were put on a diet low in AGEs presented with significantly increased insulin sensitivity after only four weeks (54).

1.5.2 Association of intake of various types of drinks and AGEs

Certain beverages such as sugar-sweetened fruit drinks and energy drinks, which are sweetened with high fructose corn syrup (HFCS), contain a high amount of AGEs (55). A Japanese study performed in 2015 studied the mean concentration of AGEs by measuring the fructose-glycation and glucose-glycation in various commonly consumed beverages and found that energy drinks and sugar-sweetened beverages (SSB) contained more units per bottle than tea and black coffee, which showed relatively low levels of AGEs (56).

The contribution to the buildup of AGEs originate from the consumption of exogenous AGEs as well as internal production that happens after the intake of sweetened beverages (57). Another study found that sugar-sweetened beverages can produce a shortening of telomeres on chromosomes, which eventually leads to cell damage, accelerated cell aging and further down the line also metabolic changes (58, 59). Figure 5 presents immediate effects of intake of SSB, which leads to a high fructose intake, high glycemic load and a rapid insulin response (59). The biological changes due to SSB intake lead to metabolic changes, such as insulin resistance, oxidative stress and inflammation. Later down the line, a telomeric shortening can be found as well as metabolic disease progression, such as hypertension, dyslipidemia, metabolic syndrome development, cardiovascular disease and type 2 diabetes (58, 60).

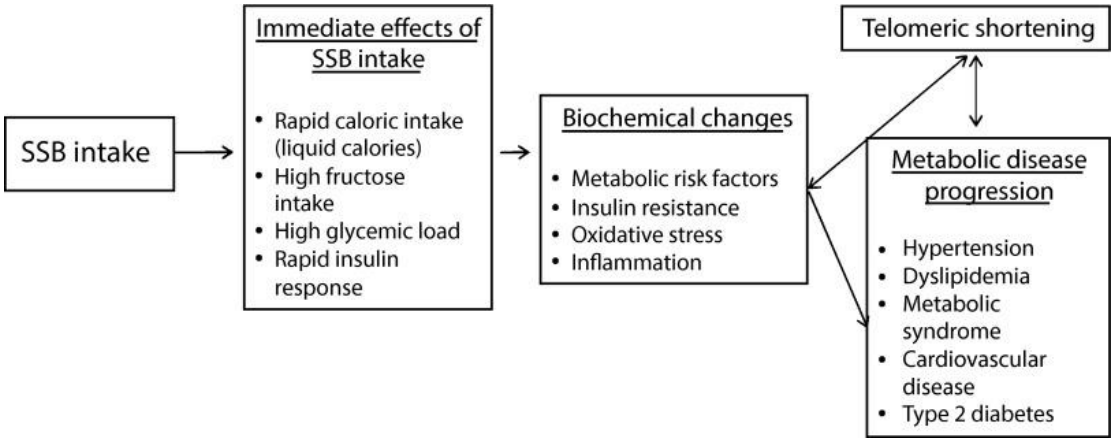

Figure 5. A model of the effects of SSBs intake on telomeric shortening and metabolic disease progression (Available from: 58)

Naturally occurring antioxidants are important scavengers of free radicals and for the reduction of AGEs. Antioxidants found in many fruits and vegetables, as well as in coffee, black tea and red wine, are compounds called polyphenols, phenolics and flavonoids (61). Phenolic acids have been widely studied for their function in health due to their antioxidant, antitumor and anti-inflammatory properties (62). Studies have proposed that the consumption of these sources can become one of the main recommendations in the prevention of diseases developing in the presence of oxidative stress, such as cardiovascular disease and diabetes mellitus (63, 64). As well as prevent the formation of toxic compounds through the Maillard reaction (65).

Coffee, being common and spread across the globe, has been widely studied for its polyphenol content and many health benefits. It has been shown to maintain a high level of antioxidants in 100% Arabica coffee beans, while the degree of roasting must be monitored carefully (66). The origin of the coffee beans also plays a role in quality and antioxidant levels (67). A lighter roasted coffee contains higher amounts of polyphenols and antioxidant levels (68). On the other hand, when darkly roasted and consumed in high amounts, coffee was found to be associated with an increased skin autofluorescence and furthermore an accumulation of AGEs (27). A study performed in 2015 concluded that a higher caffeine intake contributed to skin intrinsic fluorescence (SIF) in individuals with diagnosed diabetes, hence it should be further studied with the aspect of being a risk factor for CVD in these patients (69). Others concluded that habitual caffeine intake of 230 ml on an average of four times per day caused

adverse effects on glucose metabolism leading to an exaggerated postprandial glucose response in patients with type 2 diabetes (70).

2. Aim and Hypothesis

2.1 Aim

The aim of this thesis is to investigate the association between AGEs and various types of drinks, such as coffee, tea, wine, SSB drinks and energy drinks, in a healthy population of students.

2.2 Hypotheses

1. Students who consume coffee more frequently have higher values of AGEs in their skin compared to student who consume coffee less frequently during the week
2. Students who consume tea more frequently have lower values of AGEs in their skin compared to student who consume tea less frequently during the week
3. Students who consume wine more frequently have lower values of AGEs in their skin compared to student who consume wine less frequently during the week
4. Students who consume sugar-sweetened drinks more frequently have higher values of AGEs in their skin compared to student who consume sugar-sweetened drinks less frequently
5. Students who consume energy drinks more frequently have higher values of AGEs in their skin compared to student who consume energy drinks less frequently

3. Materials and methods

3.1. Study design

This is a cross-sectional study.

3.2. Subjects

During the period of March 2019 until December 2019, we have sampled students from two faculties from the University of Split. We have included medical students from first (N=87), fifth (N=72) and sixth (N=66) study year, with the corresponding response rates of 96.6%, 90.0% and 95.6%. Additionally, we sampled students from the University Department of Health Studies, both undergraduate and graduate level students from all three study years, including nurses, physiotherapists, lab technicians and radiology technicians, with the overall sample of 320 students (response rate of 81%).

After excluding subjects for whom we didn't obtain the AGEs measurement or they didn't respond to the questionnaire, we included 516 students into the final sample (219 medical students and 297 students from health studies).

The study was approved by the Ethical Committee of the University of Split School of Medicine (2181-198-03-04-18-0027) and the Ethical Committee of the University Department of Health Studies (2181-228-07-19-0021).

3.3 Questionnaire

The questionnaire was anonymous, and it was self-administered and paper based. It included questions on age, gender, study program, body weight, height, the number of days since last weighed, and the habits. There was a section on dietary pattern, smoking and sedentary types of physical activity.

The part of the questionnaire on dietary pattern included questions on the number of days in the week students eat breakfast, frequency of consumption of fruit and vegetables, and a question on their snacking habits while watching TV or studying (possible answers were: often, sometimes and no).

The students were asked whether they are active smokers, or they have quit smoking some time ago (ex-smokers) or they never smoked (non-smokers).

Sedentary types of physical activity included average sitting time during the day expressed in hours, alongside with the TV watching time daily, computer use daily, and mobile use daily.

We also asked student to rate their health on a Likert scale, where a zero indicated poor health (being very sick) and 10 represented best possible health.

Body mass index (BMI) was calculated using the formula:

$$\text{BMI}= \frac{weight\ (kg)}{height^2\ (m^2)}$$

3.3.1 Dietary habits and consumption of various types of drinks

The frequency of consumption of fruit, vegetables (all types) and various types of drinks was part of the questionnaire. Questions on drinks included coffee, tea, wine, sugar-sweetened drinks and energy drinks.

Possible answers to all the food and drink items were:

1. each day, twice or more a day
2. each day, once a day
3. 3 times a week
4. 2 times a week
5. once a week
6. once a month
7. rarely or never

Based on these responses, the subjects were divided in three groups: daily intake (includes responses: each day, twice or more a day and each day, once a day), weekly intake (3 times a week, 2 times a week and once a week), monthly or rarely (included answers: once a month and rarely or never).

3.3.2. AGEs measurement

AGEs measurement was performed by a simple and non-invasive approach using the AGE Mu Reader (DiagnOptics, Groningen, The Netherlands). Subjects would place the volar side of their dominant forearm on the device and the result would be available on the screen after 12 seconds. The readout would be done according to the age of the subject.

This device was validated against the skin biopsies (gold standard) in Caucasian diabetic and control subjects (71). After emitting the ultraviolet light with wavelength of 300-420 nm, fluorescence of the skin was measured in arbitrary units (AU), since many AGEs have a characteristic of fluorescence (https://www.diagnoptics.com/advanced-glycation-endproducts/measuring-ages/).

3.4. Statistical analysis

Categorical variables were described using both absolute numbers and percentages, while ordinal and numerical variables were described using median and interquartile range (due to non-normal data distribution tested by Kolmogorov–Smirnov test, with the exception of normal distribution of AGEs according to subgroups of coffee intake frequency).

Categorical variables were tested using the χ^2 test, and for the comparison on numerical variables we used Mann-Whitney U test (for comparison of two groups) and Kruskal-Wallis test (for comparison of three groups) and student's t-test (for variable with normal distribution). Correlation between numerical variables was tested with Spearman rank test.

Statistical analysis was performed with the SPSS statistical program (IBM SPSS Statistics v21), and P value of <0.05 was considered to be statistically significant.

4. Results

This study included 516 students attending medical studies and health studies at the University of Split. There were 219 medical students (42.4%), and 297 students from health studies (57.6%). Subjects' characteristics according to the study subject (faculty) are shown in Table 1. There was a statistically significant difference in gender distribution (P<0.001). Women were more frequent than men in both subsamples, but even more so among students from health studies (87.5% were women and 12.5% were men). Additionally, there was a difference in age, where medical student was on average two years older than students from health studies (P<0.001).

There was no difference in BMI, days since previous weighing, self-rated health perception, fruit intake and breakfast frequency between students from the two faculties. On the other hand, health studies students were more commonly active smokers (27.1% vs. 15.1%; P=0.001), reported more TV time (P=0.002), and more time using mobile (P=0.001), while medical students reported greater average daily sitting time (6h vs 4.5h). Daily intake of vegetables was reported by 69.4% of medical students and 56.1% of health studies students, while 0.5% of medical students and 4.4% of health studies students reported consuming vegetables monthly or less frequently (P<0.001). Health studies students also reported snacking more frequently while watching TV or studying compared to medical students (P=0.012). Moreover, health studies students had higher average AGEs values than medical student (P<0.001) (Table 1).

Table 1. Subjects' characteristics according to the study subject (faculty)

	Medical students N=219	Health studies students N=297	P
Gender; N (%)			<0.001*
Men	74 (33.8)	37 (12.5)	
Women	145 (66.2)	260 (87.5)	
Age (years); median (IQR)	23.0 (4.0)	21.0 (6.0)	<0.001#
BMI (kg/m^2); median (IQR)	22.1 (3.5)	22.0 (3.7)	0.676#
Days since previous weighing; median (IQR)	14.0 (25.0)	15.0 (26.0)	0.909#
Sitting time (h/d); median (IQR)	6.0 (3.0)	4.5 (4.0)	<0.001#
TV time daily (h/d); median (IQR)	0.8 (1.0)	1.0 (1.5)	0.002#
Computer time daily (h/d); median (IQR)	1.0 (1.7)	1.0 (1.5)	0.032#
Mobile time daily (h/d); median (IQR)	3.0 (3.0)	4.0 (3.3)	0.001#
Self-rated health; median (IQR)	9.0 (1.0)	8.0 (1.0)	0.323#
Smoking; N (%)			0.001*
Nonsmokers	154 (75.1)	173 (58.6)	
Ex-smokers	20 (9.8)	42 (14.2)	
Active smokers	31 (15.1)	80 (27.1)	
Breakfast; N (%)			0.101*
6-7 days per week	130 (63.7)	157 (54.1)	
3-5 days per week	48 (23.5)	84 (29.0)	
0-2 days per week	26 (12.7)	49 (16.9)	
Fruit intake; N (%)			0.814*
Daily	132 (60.3)	171 (57.6)	
Weekly	82 (37.4)	118 (39.7)	
Monthly or rarely	5 (2.3)	8 (2.7)	
Vegetables intake; N (%)			<0.001*
Daily	152 (69.4)	166 (56.1)	
Weekly	66 (30.1)	117 (39.5)	
Monthly or rarely	1 (0.5)	13 (4.4)	
Snacking while watching TV or studying; N (%)			0.012*
Yes, often	38 (17.4)	66 (22.3)	
Yes, sometimes	111 (50.9)	170 (57.4)	
No	69 (31.7)	60 (20.3)	
AGEs; median (IQR)	1.4 (0.3)	1.5 (0.5)	<0.001#

IQR- interquartile range, BMI – body mass index, AGEs – Advanced Glycation End products; *χ^2 test; #Mann-Whitney U test

Table 2. present the findings on the consumption of various drinks, testing for the differences between medical students and health studies students. A statistical significance was found in daily tea intake where medical students had a higher intake frequency than students in health studies (P=0.009). Health studies students also reported a higher intake of sugar-sweetened drinks than medical students did (P=0.016). No statistical significance was found regarding coffee intake, as both groups presented similar intake, nor was there any difference in wine and energy drinks consumption.

Table 2. Consumption of various drinks, according to the study subject (faculty)

	Medical students N=219	Health studies students N=297	P*
Coffee intake; N (%)			0.785
Daily	138 (63.9)	188 (63.7)	
Weekly	31 (14.4)	48 (16.3)	
Monthly or rarely	47 (21.8)	59 (20.0)	
Tea intake; N (%)			
Daily	60 (27.5)	56 (18.9)	0.009
Weekly	97 (44.5)	123 (41.4)	
Monthly or rarely	61 (28.0)	118 (39.7)	
Wine intake; N (%)			0.696
Daily	5 (2.3)	10 (3.4)	
Weekly	53 (24.4)	66 (22.5)	
Monthly or rarely	159 (73.3)	217 (74.1)	
Sugar-sweetened drinks; N (%)			0.016
Daily	41 (19.9)	93 (31.4)	
Weekly	99 (48.1)	119 (40.2)	
Monthly or rarely	66 (32.0)	84 (28.4)	
Energy drinks; N (%)			0.259
Daily	1 (0.5)	6 (2.0)	
Weekly	17 (7.8)	27 (9.2)	
Monthly or rarely	201 (91.8)	262 (88.8)	

*χ^2 test

Subjects' characteristics according to gender is shown in Table 3. Comparing the two groups there was no statistical significance in age, sitting time (h/d), TV time daily and self-rated health perception. However, a statistical significance was found for BMI, where men had an average BMI of 23.9 kg/m^2 compared to 21.6 kg/m^2 in women ($P<0.001$). There was also a significance in days since previous weighing ($P=0.008$). Additionally, men had a higher computer time daily ($P<0.001$), whereas women spent more time on the phone ($P<0.001$). Both men and women reported similar percentage of active smokers (22%), but men reported a higher percentage of students who never smoked (73% vs 63%). No statistical significance was found in breakfast frequency, fruit and vegetables intake, snacking while watching TV and in AGEs between men and women (Table 3).

Table 3. Subjects' characteristics according to the gender

	Men N=111	Women N=405	P
Age (years); median (IQR)	23.0 (5.0)	21.0 (5.0)	0.101#
BMI (kg/m^2); median (IQR)	23.9 (3.2)	21.6 (3.3)	<0.001#
Days since previous weighing; median (IQR)	10.0 (27.0)	15.0 (25.0)	0.008#
Sitting time (h/d); median (IQR)	6.0 (3.0)	5.0 (4.0)	0.078#
TV time daily; median (IQR)	1.0 (2.0)	1.0 (1.4)	1.000#
Computer time daily; median (IQR)	1.0 (2.0)	1.0 (1.5)	<0.001#
Mobile time daily; median (IQR)	3.0 (2.0)	3.5 (3.0)	0.001#
Self-rated health; median (IQR)	9.0 (1.0)	8.0 (1.0)	0.394#
Smoking; N (%)			0.010*
Nonsmokers	77 (73.3)	250 (63.3)	
Ex-smokers	4 (3.8)	58 (14.7)	
Active smokers	24 (22.9)	87 (22.0)	
Breakfast; N (%)			0.937*
6-7 days per week	62 (59.6)	225 (57.7)	
3-5 days per week	27 (26.0)	105 (26.9)	
0-2 days per week	15 (14.4)	60 (15.4)	
Fruit intake; N (%)			0.787*
Daily	62 (55.9)	241 (59.5)	
Weekly	46 (41.4)	154 (38.0)	
Monthly or rarely	3 (2.7)	10 (2.5)	
Vegetables intake; N (%)			0.229*
Daily	65 (58.6)	253 (62.6)	
Weekly	45 (40.5)	138 (34.2)	
Monthly or rarely	1 (0.9)	13 (3.2)	
Snacking while watching TV or studying; N (%)			0.055*
Yes, often	17 (15.3)	87 (21.6)	
Yes, sometimes	57 (51.4)	224 (55.6)	
No	37 (33.3)	92 (22.8)	
AGEs; median (IQR)	1.4 (0.4)	1.5 (0.4)	0.078#

IQR- interquartile range, BMI – body mass index, AGEs – Advanced Glycation End products; *χ2 test; #Mann-Whitney U test

Characteristics of subjects in consumption of various drinks, according to gender is given in Table 4. Statistical significance was found only for the intake of energy drinks, where women reported a higher frequency of daily intake than men (P=0.002). No difference was found regarding intake of coffee, tea, wine and sugar-sweetened drinks, which were all similar in both groups.

Table 4. Consumption of various drinks, according to the gender

	Men N=219	Women N=297	P*
Coffee intake; N (%)			0.143
Daily	64 (58.7)	262 (65.2)	
Weekly	15 (13.8)	64 (15.9)	
Monthly or rarely	30 (27.5)	76 (18.9)	
Tea intake; N (%)			0.161
Daily	23 (20.7)	93 (23.0)	
Weekly	41 (36.9)	179 (44.3)	
Monthly or rarely	47 (42.3)	132 (32.7)	
Wine intake; N (%)			0.125
Daily	6 (5.5)	9 (2.3)	
Weekly	21 (19.1)	98 (24.5)	
Monthly or rarely	83 (75.5)	293 (73.3)	
Sugar-sweetened drinks; N (%)			0.940
Daily	29 (27.6)	105 (26.4)	
Weekly	46 (43.8)	172 (43.3)	
Monthly or rarely	30 (28.6)	120 (30.2)	
Energy drinks; N (%)			0.002
Daily	0 (0.0)	7 (1.7)	
Weekly	18 (16.5)	26 (6.4)	
Monthly or rarely	91 (83.5)	372 (91.9)	

*χ^2 test

Subjects' characteristics according to smoking habits are shown in Table 5. Ex-smokers were on average older than smokers and non-smokers (P=0.001), non-smokers had the highest self-rated health perception (P=0.030), most frequently consumed breakfast (P=0.025) and had the lowest average AGEs accumulation (P<0.001). There were no differences in BMI, days since previously weighing, sitting time (h/d), intake of fruit and vegetables, and snacking while watching TV or studying (Table 5).

Table 5. Subjects' characteristics according to the smoking habits

	Smokers N=111	Ex-smokers N=62	Non-smokers N=327	P
Age (years); median (IQR)	22.0 (5.0)	23.0 (10.8)	21.0 (5.0)	0.001†
BMI (kg/m^2); median (IQR)	22.3 (2.9)	22.2 (4.7)	21.9 (3.9)	0.083†
Days since previous weighing; median (IQR)	15.0 (25.0)	10.0 (27.0)	14.0 (26.0)	0.340†
Sitting time (h/d); median (IQR)	5.0 (4.0)	4.3 (4.9)	5.0 (3.0)	0.177†
Self-rated health; median (IQR)	8.0 (2.0)	8.0 (2.5)	9.0 (1.0)	0.030†
Breakfast; N (%)				0.025*
6-7 days per week	52 (48.1)	31 (50.8)	201 (62.4)	
3-5 days per week	31 (28.7)	21 (34.4)	80 (24.8)	
0-2 days per week	25 (23.1)	9 (14.8)	41 (12.7)	
Fruit intake; N (%)				0.230*
Daily	56 (50.5)	41 (66.1)	194 (59.3)	
Weekly	51 (45.9)	19 (30.6)	127 (38.8)	
Monthly or rarely	4 (3.6)	2 (3.2)	6 (1.8)	
Vegetables intake; N (%)				0.851*
Daily	66 (59.5)	41 (66.1)	197 (60.4)	
Weekly	41 (36.9)	20 (32.3)	121 (37.1)	
Monthly or rarely	4 (3.6)	1 (1.6)	8 (2.5)	
Snacking while watching TV or studying; N (%)				0.698*
Yes, often	21 (18.9)	16 (25.8)	65 (19.9)	
Yes, sometimes	64 (57.7)	29 (46.8)	180 (55.0)	
No	26 (23.4)	17 (27.4)	82 (25.1)	
AGEs; median (IQR)	1.6 (0.5)	1.6 (0.5)	1.4 (0.3)	<0.001†

IQR- interquartile range, BMI – body mass index, AGEs – Advanced Glycation End products; *χ^2 test; †Kruskal-Wallis test

According to the results presented in Table 6. a positive correlation was found between AGEs and age ($P<0.001$), BMI ($P<0.001$), and coffee intake ($P<0.001$). Older subjects, students with a higher BMI had higher accumulation of AGEs in the body, the same as students who reported higher intake of coffee. There was no correlation between AGEs and other types of drinks, namely tea, wine, sweetened drinks and energy drinks. A negative correlation was recorded for AGEs and breakfast frequency during the week ($P=0.015$; Table 6). Breakfast was also negatively correlated with BMI ($P=0.017$), and positively correlated with fruit intake ($P=0.002$), vegetables intake ($P=0.004$), coffee ($P=0.027$) and tea intake ($P<0.001$). Self-rated health was positively correlated with fruit ($P=0.044$) and vegetables intake ($P=0.005$), and negatively with sweetened drinks ($P=0.009$; Table 6).

Table 6. Correlation between AGEs and various types of drinks and other important variables (numbers are Spearman rho coefficients; P values)

	Age	AGEs	BMI	Weighing	Breakfast	Self-rated health	Fruit	Vegetables	Sweets	Sugar-sweetened drinks	Wine	Coffee	Tea	Energy drinks
Age	-	0.405; **<0.001**	0.179; **<0.001**	-0.113; **0.012**	-0.089; **0.049**	0.004; 0.938	0.092; **0.039**	0.129; **0.004**	0.012; 0.784	-0.089; **0.046**	0.157; **<0.001**	0.173; **<0.001**	-0.046; 0.303	-0.066; 0.140
AGEs		-	0.163; **<0.001**	-0.084; 0.063	-0.110; **0.015**	-0.067; 0.141	0.040; 0.363	-0.029; 0.516	0.015; 0.729	0.054; 0.227	-0.001; 0.983	0.221; **<0.001**	0.015; 0.728	-0.031; 0.488
BMI			-	-0.101; **0.026**	-0.108; **0.017**	-0.070; 0.125	0.047; 0.291	0.012; 0.786	0.087; 0.052	-0.037; 0.417	0.024; 0.602	0.150; **0.001**	0.058; 0.195	0.140; **0.002**
Weighing				-	0.012; 0.785	-0.065; 0.160	0.075; 0.094;	-0.116; **0.010**	-0.072; 0.111	-0.041; 0.364	0.023; 0.615	0.061; 0.179	-0.014; 0.758	0.071; 0.118
Breakfast					-	0.063 0.169	0.141; **0.002**	0.131; **0.004**	-0.001; 0.986	-0.082; 0.071	0.076; 0.095	0.100; **0.027**	0.177; **<0.001**	0.033; 0.459
Self-rated health						-	0.092; **0.044**	0.128; **0.005**	0.046; 0.313	-0.119; **0.009**	0.020; 0.668	0.014; 0.758	0.021; 0.649	-0.019; 0.684
Fruit consumption							-	0.456; **<0.001**	-0.017; 0.696	-0.096; **0.031**	0.021; 0.637	0.026; 0.562	0.268; **<0.001**	-0.025; 0.565
Vegetables consumption								-	-0.121; **0.006**	-0.259; **<0.001**	0.140; **0.001**	0.097; **0.028**	0.204; **<0.001**	-0.043; 0.330
Sweets									-	0.286; **<0.001**	-0.025; 0.578	0.102; **0.022**	-0.017; 0.695	-0.003; 0.938
Sugar-sweetened drinks										-	0.036; 0.428	-0.043; 0.341	-0.048; 0.279	0.191; **<0.001**
Wine											-	0.199; **<0.001**	0.140; **0.002**	0.153; **0.001**
Coffee												-	0.118; **0.007**	0.039; 0.381
Tea													-	0.110; **0.012**

Figure 6 displays the means for AGEs values, according to the groups of students with different coffee intake frequency, with clear increase in AGEs with the increasing frequency of coffee intake. The value of mean for AGEs in students who never consumed coffee was 1.46±0.32, while students who consumed coffee ≥2 times a day had the average AGEs value of 1.62±0.36 (t test P<0.001).

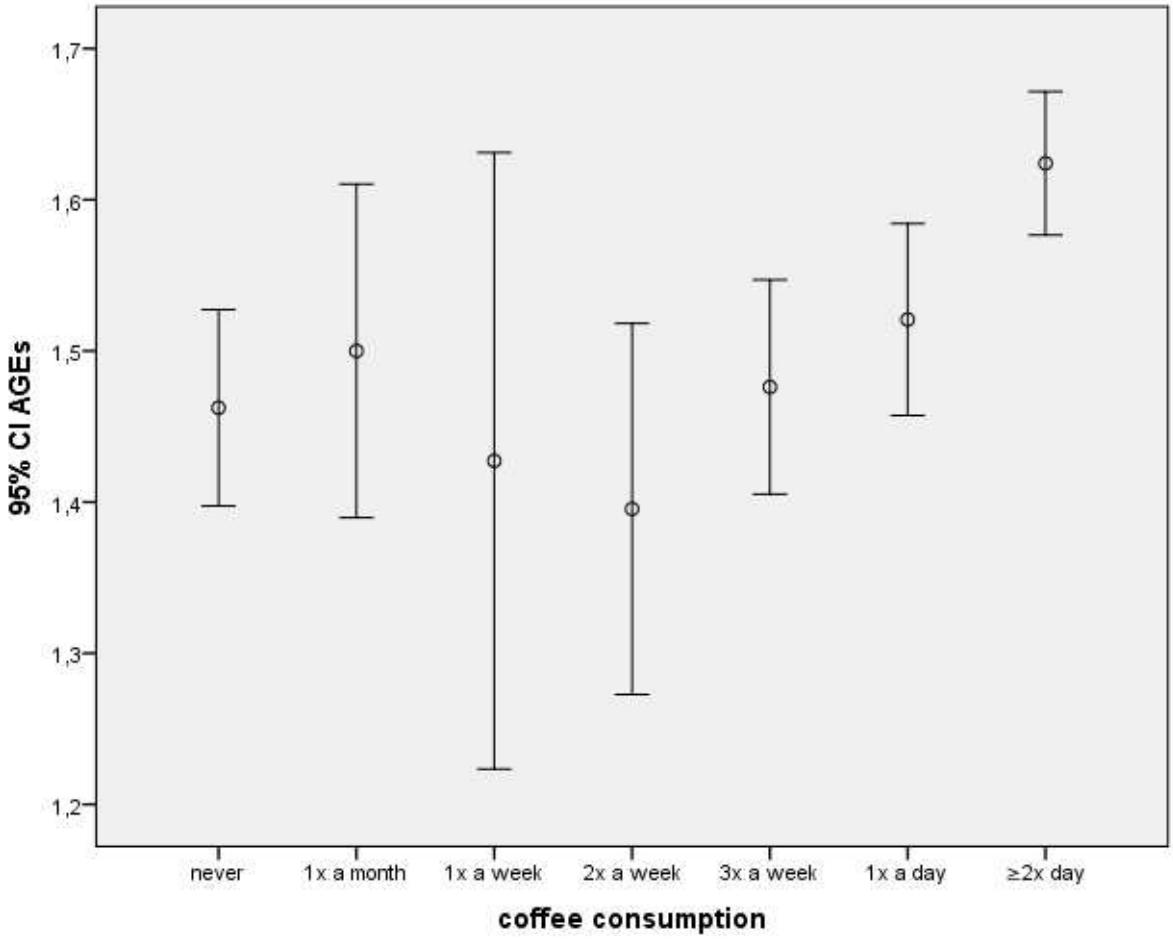

Figure 6. Average values of AGEs according to the coffee intake frequency (circles are means and bars represent 95% CI of the mean). Note: data were distributed normally within the subgroups of coffee intake.

5. Discussion

The results of the presented study confirm our hypothesis that students who consume coffee more frequently have higher values of AGEs in their skin compared to students who consume coffee less frequently.

This finding of positive association between coffee intake and AGEs run in line with a few previous studies from the literature. For example, a cross-sectional study performed with 957 adult participants from the general Dutch population found an association between skin autofluorescence and an increased coffee intake of 50 ml per day, after adjustment for age, smoking status, educational level and CVD (27). SAF also increased with an increasing chronological age. Furthermore, they found a large difference in AGEs between men and women, where men had a higher risk of an increased SAF than women, suggesting that separate measures should be made for both groups (27). A study which used data from LifeLines Cohort study, with a large sample size, concluded that a dose-dependent intake of coffee was associated with higher SAF levels, in both a diabetic and non-diabetic population (12, 73). Eny *et al*. found that caffeine consumption also correlated with SIF in participants with type 1 diabetes (69).

Coffee has been suggested to contribute to AGEs formation through the Maillard reaction during the process of roasting the coffee beans at 180-250 °C. This is done to extract the sought-after flavor and fragrance. However, it also leads to formation of melanoidins (67). It is also suggested that caffeine possess fluorescent properties called fluorophores (74). This indicates that the consumption of coffee probably has a correlation with AGEs through both internal formation, as well as exogenous intake of AGEs. In addition to the increase of AGEs in correlation to coffee intake, we also found that coffee drinkers had a higher BMI than those who had a lower coffee consumption.

Results from other studies showed that an increase in skin autofluorescence correlate with an increased amount of AGEs, which in turn indicates a higher chance of developing chronic diseases, such as diabetes mellitus and cardiovascular disease (32, 33). However, the use of SAF in the prediction of cardiovascular events may lead to an overestimation in subjects who have a higher coffee consumption, since coffee has been shown to affect the SAF in a dose-dependent manner (69). This is significant due to the reason that coffee intake is probably not associated with chronic diseases themselves, but rather gives us high values due to an elevation in SAF.

Contrary to our expectation, we didn't find association between AGEs and SSB intake. It needs to be added that not all SSBs have the same amount of AGEs nor level

of sweetener added, and it would be suggestive to assume that a higher amount of sugar also comes with higher level of AGEs. Furthermore, another study found that the amount of AGEs ingested in a serving of regular cola contained <2% of daily urinary output of AGEs, suggesting that cola drinks contain low amount of glycation adduct and might also have low bioavailability (44, 72). Still, a large cohort study, which included men and women in the ages of 45-70 years with no previous history of cardiovascular risk factors, concluded that a low intake of SSB was associated with a decreased SAF (50).

Although there are no previous reports on energy drinks, we did hypothesize that energy drinks would increase AGEs in the skin due to their sugar and caffeine content, it was surprisingly not a factor associated with AGEs, even though intake of energy drinks was positively associated with BMI. One possible explanation for the lack of association found between energy drinks and AGEs is a low frequency of intake of these type of drinks in our student population.

We also failed in confirming our other hypotheses, where we expected lower AGEs accumulation in students with greater tea and wine intake. Even though daily tea intake was less common than coffee intake, it was significantly correlated with coffee intake, and wine was seldom consumed on a daily basis in our sample, which could explain our null findings. Even though there is a lack of similar studies in the literature, one study found that red wine contains high amounts of polyphenols and flavonoids and when consumed in moderation it can have anti-glycation and protective effects for cardiovascular events (50). Wu *et al*. found that red wine contained resveratrol, a polyphenol, and when consumed in moderation had cardioprotective effects by minimizing the progression of atherosclerosis in rabbits on a high-fat diet (64). Tea was also found to contain only low amount of AGEs (56). It might be reasonable in future studies to narrow down and control the type of tea consumed, as well as limiting wine intake to red wine and monitor more precise volumes consumed.

We found that students attending health studies, subjects who smoked (active smokers and ex-smokers), those who consumed breakfast less regularly and subjects with a higher BMI presented with a greater accumulation of AGEs in their skin. These findings are in line with many previous studies, one of which was performed in 2019, with a focus on modern American diet high in AGEs and health outcomes (4). Where women with polycystic ovary syndrome and a BMI over 25 kg/m^2 consumed a high-AGE diet. They found that AGEs had effects not only on the cardiovascular system,

but also mimicked hormones and modulated their receptors on a DNA level, leading to high levels of androgens and insulin, among others, to promote metabolic and ovarian dysfunction, eventually leading to infertility (4).

Our study confirmed that students who are either active smokers or ex-smokers have an increased concentration of AGEs in the skin, when compared to the group of students who never smoked. Smoking has also been taken into account in previous studies, and it has been found to positively correlate as an independent factor on SAF accumulation (8). And, also acting as a trigger for oxidative stress (9).

Sanchez *et al.* recently established a relationship between Mediterranean diet and AGEs concentration, where they showed that good adherence to a Mediterranean diet, primarily focused on the intake of olive oil, wine, fruits, vegetables, fish, legumes and nuts, can help decrease the SAF and AGEs stored in the body and therefore lower the risk of cardiovascular disease and complications (50). This runs in line with our findings on breakfast, indicating that daily breakfast consumption had a negative correlation with the concentration of AGEs and age, as well as with BMI, while there was a positive correlation between breakfast frequency and fruit, vegetables and coffee intake. On the other hand, fruit and vegetables intake was not correlated with AGEs in our study.

There are several limitations to this study, including the lack of precision on the type and the amount of drinks consumed (for SSB and energy drinks group, type of the tea), along with their unfamiliar content of sugar. The sample included only young and healthy subjects. Still, this is the first study so far to investigate the association between energy drinks and wine and AGEs accumulation in the skin, in a relatively large sample size.

6. Conclusion

Intake of 2 or more cups of coffee per day was associated with a higher value of AGEs measured in the skin of young and generally healthy people. This study failed in finding the association between AGEs accumulation and other types of drinks, namely tea, wine, sugar-sweetened drinks and energy drinks.

According to the growing body of literature, increased AGEs accumulation can be used as a marker for higher risk of developing metabolic aberrations. However, the increase of AGEs due to coffee intake might be a false elevation of AGEs assessment and should be taken into account when evaluating patients with chronic diseases through AGEs measurements. This topic should be studied further.

Furthermore, a healthy lifestyle, with a daily breakfast intake and lower BMI, alongside with previously recognized helpful Mediterranean dietary pattern, can be encouraged to a general population to improve their health and reduce the AGEs accumulation in the body.

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8. Summary

Aim: An increase in skin autofluorescence (SAF), which is used for assessing the accumulation of the advanced glycation end products (AGEs), has been found to be associated with the development of many chronic diseases. The aim of this study was to evaluate the association between AGEs and intake of different types of beverages in a healthy population of students.

Materials and methods: We included 516 students, attending medical studies (N=219) and health studies (N=297) at the University of Split, in this cross-sectional study. Students have filled out anonymous questionnaire about their habits, including food and drinks intake and we measured AGEs in the skin of the dominant forearm using a non-invasive AGE Reader device (DiagnOptics, Groningen, Netherlands). Data were analyzed using chi-square test, Mann-Whitney U test, Kruskal-Wallis test, student's t-test and Spearman rank test.

Results: Intake of coffee was associated with a higher value of AGEs (rho=0.221, P<0.001). We found no correlation between AGEs accumulation and other types of drinks, namely tea, wine, sugar-sweetened drinks or energy drinks. Additionally, AGEs were positively correlated with age (rho=0.405, P<0.001) and BMI (rho=0.163, P<0.001), and negatively correlated with the frequency of breakfast intake (rho=-0110, P=0.015). Subjects who smoked (both active smokers and ex-smokers) had on average higher levels of AGEs in their skin compared to non-smokers (P<0.001). Students attending health studies compared to medical students displayed higher levels of AGEs (P<0.001).

Conclusion: The increase in SAF due to coffee intake might be a spurious finding of AGEs accumulation, and coffee intake should be taken into account as a confounding factor when evaluating patients with chronic diseases. Furthermore, a healthy lifestyle, with a daily breakfast intake and a lower BMI, can be recommended to reduce the AGEs accumulation in the body and improve overall health.

9. Croatian summary

Naslov: POVEZANOST IZMEĐU KRAJNJIH PRODUKATA GLIKACIJE I RAZLIČITIH VRSTA PIĆA U ZDRAVOJ POPULACIJI STUDENATA

Cilj: Pokazalo se da je porast autofluorescencije kože (engl. *skin autofluorescence*, SAF), koji se koristi za procjenu nakupljanja krajnjih produkata glikacije (engl. *advanced glycation end products*, AGEs), povezan s razvojem mnogih kroničnih bolesti. Cilj ove studije bio je procijeniti povezanost između AGEs-a i unosa različitih vrsta pića u zdravoj populaciji studenata.

Materijali i metode: U ovaj presječnoj studij uključili smo 516 studenata medicine (N = 219) i zdravstvenih studija (N = 297) sa Sveučilišta u Splitu, a studenti su ispunili anonimni upitnik o svojim navikama, uključujući konzumaciju različitih skupina namirnica i unos različitih vrsta pića te smo izmjerili AGEs u koži dominantne podlaktice pomoću neinvazivnog AGE Reader uređaja (DiagnOptics, Groningen, Nizozemska). Podaci su analizirani uporabom hi-kvadrat testa, Mann-Whitney U testa, Kruskal-Wallisov testa, studentovog t-testa i Spearmanovog testa korelacije.

Rezultati: Unos kave bio je povezan s većom vrijednošću AGEs-a (rho=0,221; P<0,001). Nismo pronašli povezanost između nakupljanja AGEs-a i ostalih vrsta pića (čaja, vina, napitaka zaslađenih šećerom ili energetskih pića). Uz to, AGEs su bili pozitivno korelirani s dobi (rho=0,405; P<0,001) i indeksom tjelesne mase (rho= 0,163; P<0,001), a negativno su korelirali s učestalošću konzumacije doručka (rho=-0110; P=0,015). Ispitanici koji su pušili (i aktivni pušači i bivši pušači) imali su u prosjeku višu razinu AGEs-a u koži u usporedbi s nepušačima (P<0,001). Studenti zdravstvenih studija imali su u prosjeku višu razinu AGEs-a u usporedbi sa studentima medicine (P<0,001).

Zaključak: Povećanje SAF-a zbog unosa kave mogao bi biti djelomično lažan nalaz u prilog nakupljanja AGEs-a, tako da unos kave treba uzeti u obzir kao zbunjujući čimbenik prilikom kliničke procjene bolesnika s kroničnim bolestima. Nadalje, možemo preporučiti zdrav način života, uz svakodnevno doručkovanje i niži indeks tjelesne mase, kako bi se smanjilo nakupljanje AGEs-a u tijelu i općenito poboljšalo zdravlje.

10. Curriculum Vitae

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