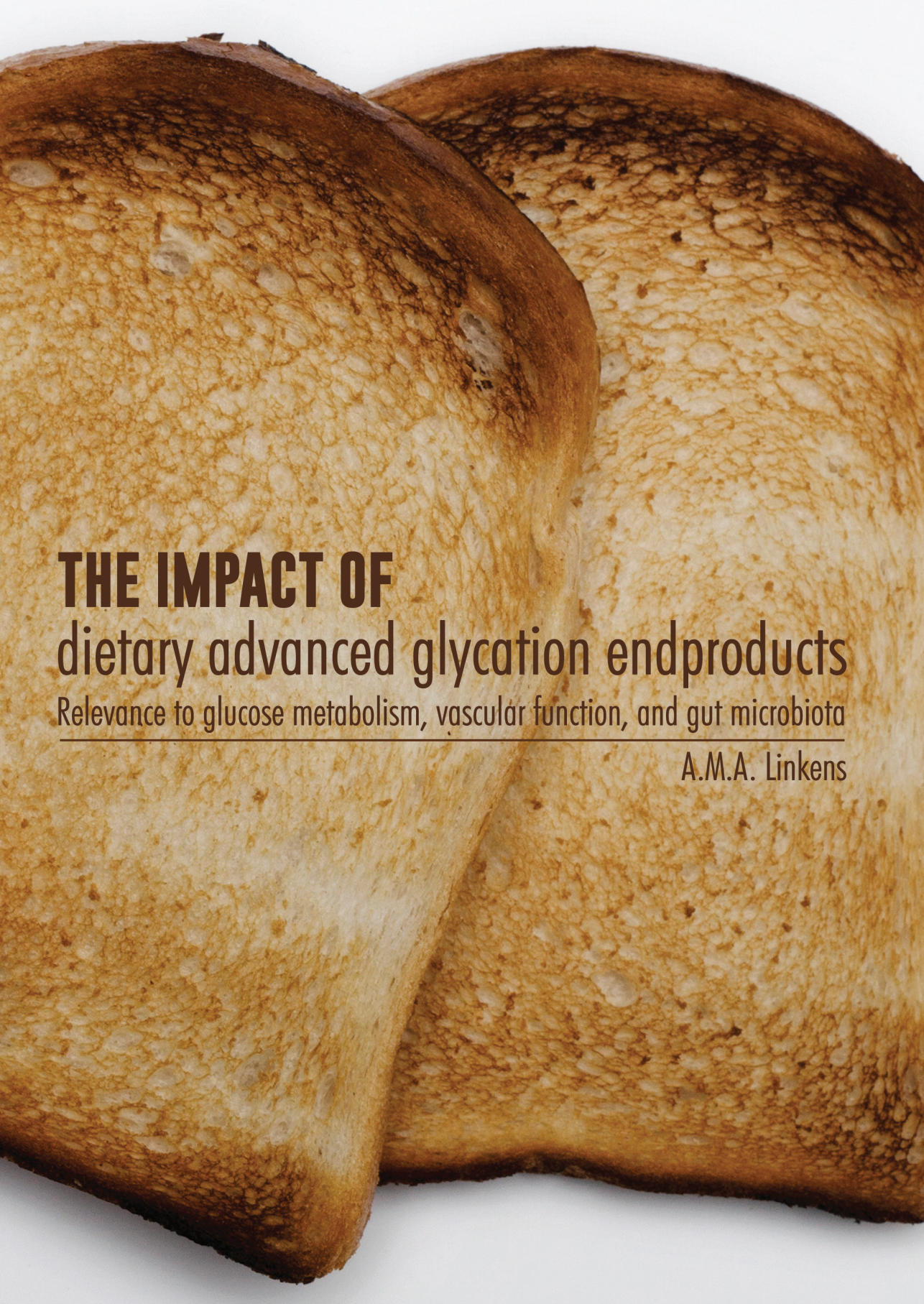
The Impact Of Dietary Advanced Glycation Endproducts



THE IMPACT OF
dietary advanced glycation endproducts
Relevance to glucose metabolism, vascular function, and gut microbiota

A.M.A. Linkens

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Armand Martinus Antoinetta Linkens

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Armand Martinus Antoinetta Linkens

Promotor
Prof. dr. C.G. Schalkwijk
Prof. dr. C.D.A. Stehouwer

Copromotoren
Dr. A.J.H.M. Houben
Dr. S.J.M.P. Eussen

Beoordelingscommissie
Prof. dr. R.P. Mensink (voorzitter)
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Prof. dr. M.C. Zillikens (Erasmus MC)

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dietary AGEs in MIXED NUTS (SALTED PEANUTS, COCKTAIL NUTS, SUGARED NUTS)

CML: 3.3; CEL 5.6; MG-H1: 38.9 mg/100g

Chapter 1

General Introduction

1.1 Obesity, type 2 diabetes mellitus, and cardiovascular disease

Obesity's worldwide prevalence has nearly tripled since 1975[1]. In line with this, the prevalence of diabetes has nearly quadrupled since 1985 (Figure 1.1), attributable mainly to an increase in type 2 diabetes mellitus (T2DM)[2]. It is estimated that in 2035 approximately 8.8% of the world's population aged 20-79 will have diabetes. In the Netherlands, this number is estimated at a similar 9.0%[3]. Diabetes' burden is largely determined by its micro- and macrovascular complications. In fact, cardiovascular disease is the leading cause of death in patients with T2DM, accounting for 52% of total cases[4]. Interestingly, impaired vascular function, which is often used as a surrogate of cardiovascular endpoints, is already present in the microvascular[5, 6] and macrovascular[7, 8] bed of those with prediabetes and may in fact contribute to development of T2DM[9]. As such, preventing T2DM and its vascular complications, as well as protecting vascular function in those at risk, is paramount. However, 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 of T2DM and its associated impairment in vascular function.

Since it is unlikely that the genetic make-up of humankind has changed significantly in the past decades, it is generally accepted that a change in diet is a key determinant of the T2DM epidemic. In particular, consumption of processed foods, sugar, and fat has increased[10] and is associated with insulin resistance[11], which in turn is crucial in the development of T2DM[12] and impaired vascular function[13]. Processed food has often been exposed to heat and as a result contains advanced glycation endproducts (AGEs). Among other factors, AGEs in food are potential risk factors for insulin resistance and T2DM. A diet high in these dietary AGEs has been linked to insulin resistance, low-grade inflammation, and endothelial dysfunction[14], and could contribute to T2DM's rising prevalence. As such, reducing AGE intake has already been suggested as a treatment strategy to prevent T2DM. However, studies so far yielded inconsistent results, are characterized by common methodological limitations, and the underlying mechanisms are incompletely understood. Thus, it is still uncertain whether dietary AGEs contribute to insulin resistance and the development of T2DM, and to vascular disease. Therefore, the aim of this thesis is to investigate with state-of-art techniques whether dietary AGEs are involved in the development of T2DM and impaired vascular function, as well as to study the underlying pathophysiological mechanisms.

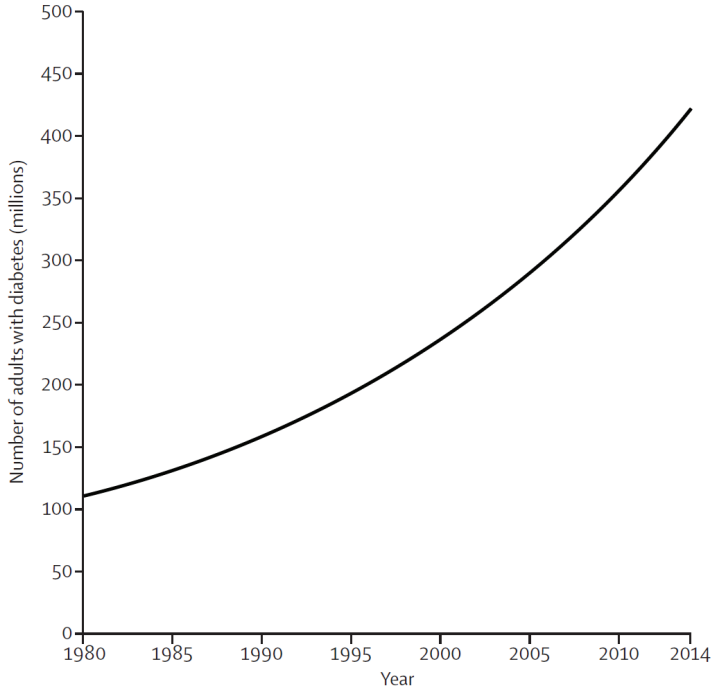


Figure 1.1 Worldwide increase in diabetes prevalence from 1980-2014. Adapted from[15].

1.2 Type 2 diabetes mellitus: role of insulin sensitivity and secretion

T2DM results from decreased insulin sensitivity (insulin resistance) and inadequate insulin secretion (beta-cell dysfunction). To regulate plasma glucose levels in the postprandial state pancreatic beta-cells secrete insulin, which serves distinct metabolic and vascular functions. On the one hand, insulin induces translocation of the GLUT-4 transporter to the cellular membrane of myocytes and adipocytes, enabling glucose uptake from the circulation[16]. On the other hand, insulin increases blood flow to enhance delivery of glucose and insulin to peripheral tissues in a process called capillary recruitment[17]. Via a variety of independent pathways[18] including adipose tissue expansion[19], intrahepatic lipid accumulation[20], and reduced capillary recruitment[21], insulin's potency to elicit these functions is decreased and insulin resistance occurs. Typically, insulin resistance precedes development of T2DM, and euglycemia is initially maintained by adaptations in beta-cell function and increased secretion of insulin. Over time, progressive insulin resistance renders adaptations in beta-cell function inadequate and hyperglycemia, and thus prediabetes and eventually T2DM, ensues[22].

1.3 Advanced glycation endproducts

AGEs are a heterogeneous group of bioactive compounds that are most known from their in vivo formation despite their widespread presence in food. In the body, they form from the non-enzymatic reaction between sugars and amino groups in proteins and other macromolecules such as lipids. AGEs are formed through various pathways of which the Maillard reaction is most known[23]. This reaction describes a series of steps that are initially reversible but ultimately form non-reversible AGEs. First, over the course of several hours, condensation of a sugar aldehyde's carbonyl group with the free amino group of a protein leads to formation of a so-called Schiff base. Second, over the course of several days, internal rearrangements of atoms resulting from acid-base catalysis leads to formation of the more stable Amadori products. A well-known example of an Amadori product is glycated hemoglobin, HbA1c[24]. Third, over the course of weeks, Amadori products undergo oxidation reactions resulting in irreversible formation of AGEs[25] (Figure 1.2). In addition to slow AGE formation via the Maillard reaction, AGEs also form rapidly in vivo from highly reactive byproducts of glucose metabolism called dicarbonyl compounds[26]. Several AGEs have been characterized. N^{ε}-(carboxymethyl)lysine (CML) is the most described, characterized, and studied AGE and is formed either via the Maillard reaction through the reaction of glucose with lysine or directly through reaction of the dicarbonyl glyoxal with lysine. Other frequently-studied AGEs are N^{ε}-(1-carboxyethyl)lysine (CEL) and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1). CEL is formed from the reaction of the most potent dicarbonyl, methylglyoxal, with lysine[27], while methylglyoxal's reaction with arginine forms MG-H1[28] (Figure 1.2).

AGE formation in vivo is unavoidable and their accumulation on long-lived proteins such as collagen occurs naturally with ageing[29, 30]. However, with hyperglycemia and oxidative stress, such as occurs in T2DM, this process is accelerated[26]. AGE formation results in structural and functional changes of proteins and lipids which induce biological effects via several distinct mechanisms. First, AGEs may form protein crosslinks in the extracellular matrix. A well-known example is collagen-crosslink formation by the AGE pentosidine in the vascular wall, directly influencing its structural properties and stiffening arteries[31]. Second, intracellular AGE formation by reactive dicarbonyls may influence key signaling pathways, especially in cells that take up glucose in a concentration-dependent and insulin-independent manner such as endothelial cells. In endothelial cells, AGEs reduce endothelial nitric oxide synthase (eNOS) expression due to eNOS mRNA degradation[32], but also directly quench nitric oxide (NO)[33]. Third, AGEs bind to a variety of cell-surface receptors, of which the multiligand receptor for AGEs (RAGE) is most known[34]. RAGE is expressed by several cells, including macrophages, leukocytes, endothelial cells, and smooth

muscle cells[35]. Under physiological conditions, RAGE expression by these cells is low, but in pathophysiological conditions such as inflammation and in T2DM, expression is upregulated[25]. RAGE stimulation, for example by CML, activates signaling pathways that ultimately lead to increased production of inflammatory cytokines and adhesion molecules, and increased oxidative stress[36, 37].

Via these mechanisms, AGEs contribute to the micro- and macrovascular complications of diabetes[38-44]. Furthermore, AGEs may also contribute to development of T2DM by influencing insulin sensitivity and beta-cell function. AGEs in plasma are associated with insulin sensitivity in several human studies[41, 45, 46], and AGEs directly modulate beta-cell function as a result of increased oxidative stress[47-49]. In line with a plethora of experimental data that supports these associations[41, 50-53], plasma CML was shown to be an independent predictor of incident T2DM in humans[54].

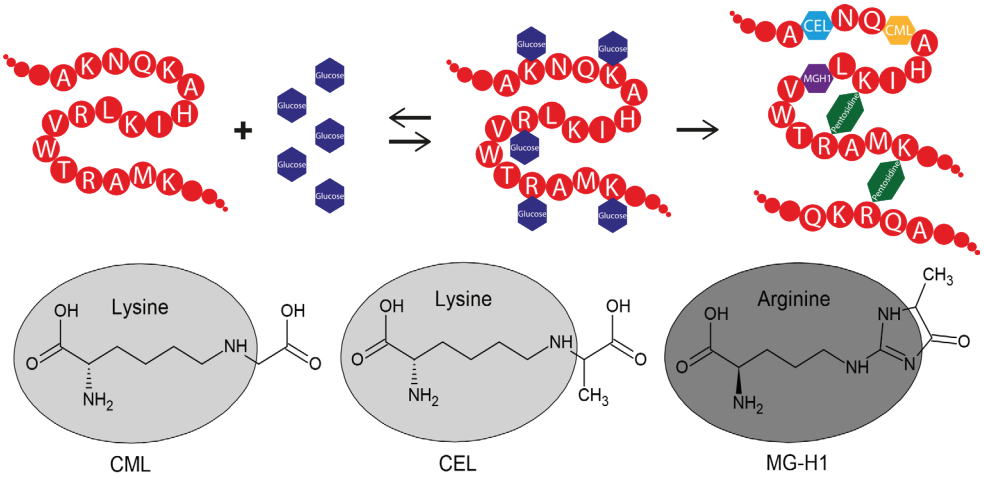


Figure 1.2 Formation of AGEs via the Maillard reaction and molecular structures of N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1).

1.4 Dietary advanced glycation end products

Aside from endogenous formation, AGEs also originate from exogenous sources such as tobacco smoke[55] and diet[56] (Figure 1.3). During food heating, commonly performed to improve flavor, color, smell, and shelf life, the rate of the Maillard reaction is accelerated. Observed visually as browning, this occurs mainly in protein- and sugar rich foods[57]. Although several factors determine the ultimate AGE quantity of foods, heat is the most potent driver. With each 10° Celsius increase in temperature AGE formation is doubled[58]. Additionally, more AGEs are formed in alkaline environments, with a maximum of pH 10[59]. Moisture is also important, as AGE formation is initially increased with rising moisture content due to enhanced mobility of reactants, while

it is ultimately decreased when the reactants are diluted[59]. Foods highest in AGEs include heat-treated processed nut- and grain products as well as canned meats[57]. Due to the contribution of endogenous AGE formation in the pathophysiology of T2DM, biological effects of a diet high in AGEs are increasingly investigated. However, due to methodological shortcomings and knowledge gaps, discussed in detail below, current knowledge does not suffice to conclude whether dietary AGEs contribute to the development of T2DM.

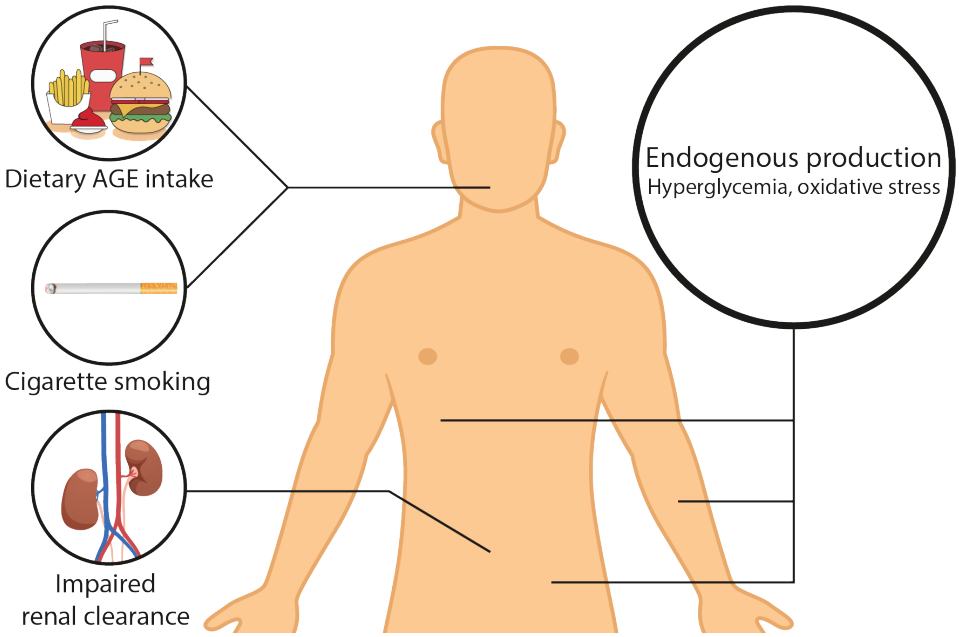


Figure 1.3 Sources of increased AGEs within the body.

1.4.1 Are dietary AGEs absorbed?

Although much is still unknown about the digestion of dietary AGEs, a combination of human and animal studies suggest that dietary AGEs are absorbed into the circulation and accumulate in tissues (Figure 1.4). In contrast to non-glycated proteins, which show digestibility of 70-90%[60], digestibility of dietary AGEs may be much less due to reduced affinity to gut proteases[61]. Early evidence for this was provided in 1997 by Koschinsky et al, who showed with enzyme-linked immunosorbent assay (ELISA) methods that 10% of dietary AGEs from a single meal were recovered in serum after 48-hours[62]. Similar results were found in mice fed ^{14}C-labeled AGEs[63]. It is hypothesized that dietary AGEs, consumed as whole proteins, undergo (partial) digestion in the gastrointestinal tract and subsequently enter the circulation as free AGEs; i.e. modified amino acids. In line with this hypothesis, we showed that greater

habitual intake of CML, CEL, and MG-H1 in humans is associated with higher levels of AGEs in plasma and urine, but only in the free amino acid form[56]. There is no direct evidence for tissue accumulation of dietary AGEs in humans. However, in mice fed a high AGE diet for 30 days, ^{13}C-labeled CML increased in several organs, including kidneys, gut, and liver[64]. Whether the accumulation of dietary AGEs in organs is reversible, which may have implications for any accumulation-associated biological effects, is currently unknown.

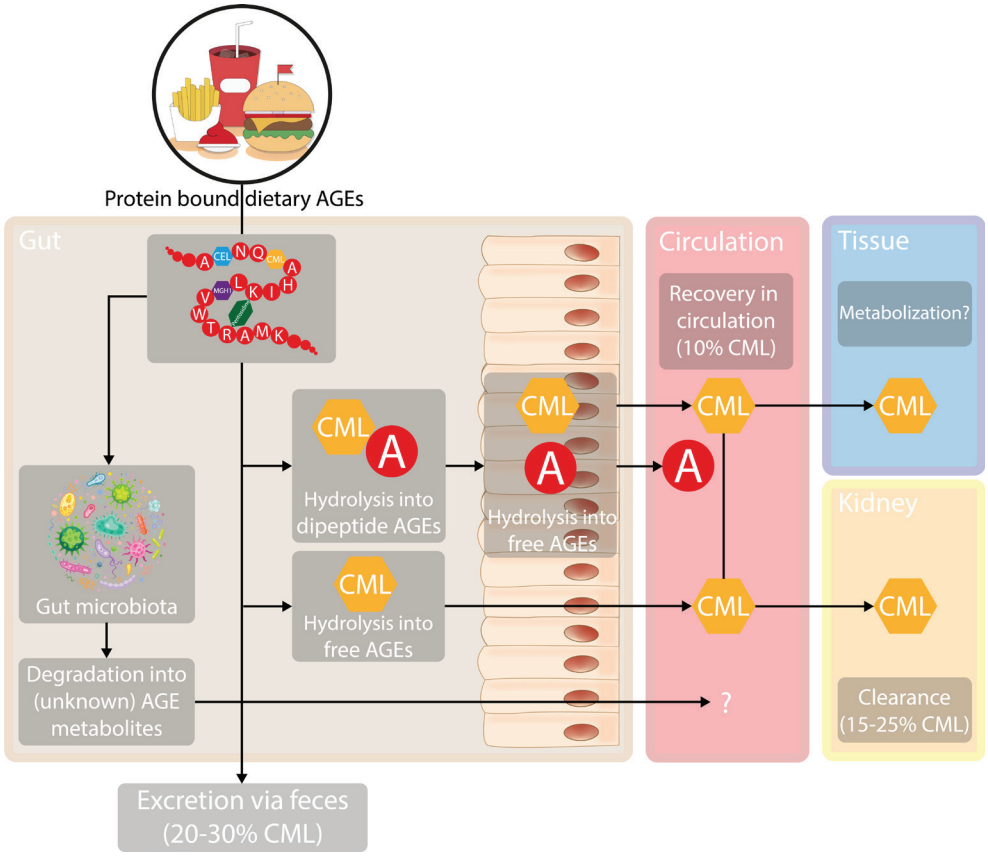


Figure 1.4 Digestion, absorption, and elimination of dietary AGEs.
CML, as the most studied dietary AGE, is shown in its protein-bound form (attached to alanine, red circle with "A") or unattached in its free form. Dietary AGEs, present in food mainly bound to proteins, are partially digested in the gut by proteases to yield AGEs bound to peptides and free amino acids. Uptake of AGEs into intestinal epithelial cells takes place in the dipeptide form, rather than the free form[65]. Intracellularly, dipeptide AGEs are hydrolyzed to yield free AGEs; i.e. modified amino acids. An estimated 10% of AGEs is recovered in the circulation[62]. In addition, certain bacterial species of the gut microbiota may degrade AGEs into unknown metabolites[66]. Whether these metabolites are absorbed or elicit biological effects are unknown. The largest proportion of dietary AGEs are excreted intact via feces (estimated 20-30%[67]). From the circulation, free AGEs are either rapidly cleared via the kidneys (estimated 15-25%[67]) or accumulate in organs. The incomplete recovery of dietary CML, which is also likely to apply to CEL and MG-H1, is suggestive of accumulation in organs and tissues but also of degradation into unknown metabolites.

1.5 Quantifying advanced glycation endproducts

AGEs can be quantified by either immunohistochemical or instrumental methods. In most studies so far AGEs were analyzed immunohistochemically by ELISA using AGE-specific antibodies. Although this method is preferred when studying distribution and localization of AGEs in tissues, its use in quantifying AGEs in different matrices shows several limitations. First, cross-reactivity of antibodies, as reported for the widely used CML antibody 6D12 with CEL, limits reliable and reproducible results[68]. Excluding cross-reactivity in general is difficult due to the large heterogeneity of AGEs. Second, a distinction between protein-bound and free amino acid AGEs cannot be made with ELISA. As explained below, this distinction is particularly important when investigating dietary AGEs. Third, ELISA yields semi-quantitative results (AGEs are expressed in arbitrary "kilounits"[69, 70]), which limits comparability between studies. To investigate biological effects of dietary AGEs, studies so far almost exclusively made use of an ELISA-based dietary AGE database covering only CML content of over 500 food products[69, 70]. Besides the reliability issues mentioned above, over- or underestimation of overall AGE intake may occur as individual AGEs in food products may vary. Furthermore, biological effects of individual dietary AGEs could vary, but this cannot be researched with this database.

These limitations are overcome when dietary AGEs are analyzed by instrumental methods such as mass spectrometry. Although the technical expertise needed to perform such analyses are greater compared to ELISA, analysis of AGEs at the molecular level yield reliable and robust quantitative data. Using ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS-MS), our group developed a new database by measuring CML, CEL, and MG-H1 in protein fractions of over 200 food items[57]. When comparing dietary AGEs between databases, several discrepancies are observed. For example, butter, mayonnaise, and olive oil, products expected to contain little AGEs due to lack of amino acids, are high in AGEs when measured with ELISA but contain virtually no AGEs when measured with UPLC-MS/MS[57, 71]. This limits the reliability of dietary AGE estimates in studies using ELISA databases. Future studies should be performed with mass spectrometry-based approaches.

1.6 T2DM, vascular function, and the role of dietary AGEs

1.6.1 Insulin sensitivity

In larger epidemiological studies, insulin sensitivity is often assessed based on fasting plasma glucose and insulin using homeostatic model assessment (HOMA)[72], or based on their plasma levels during a standard 2-hour oral glucose tolerance test using the

Matsuda index[73]. While HOMA mainly reflects hepatic insulin sensitivity, the Matsuda index also reflects peripheral insulin sensitivity. In smaller experimental trials, insulin sensitivity can be determined using the gold standard hyperinsulinemic-euglycemic clamp technique. Here, insulin is infused in a fixed dose and euglycemia is maintained by a variable glucose infusion. During steady-state conditions, glucose infusion represents whole-body glucose disposal, which largely depends on peripheral insulin sensitivity[74]. Insulin sensitivity measured by HOMA, the Matsuda index, or the clamp agree reasonably well in individuals with normal glucose metabolism (Spearman's correlation ρ 0.65 and ρ 0.73, respectively), but this noticeably decreases in individuals with impaired glucose metabolism (ρ 0.56 and ρ 0.66) and T2DM (ρ 0.51 and ρ 0.54)[75]. Because measures obtained by golden standards are the best indicators of the target concept, their use is preferred when possible.

1.6.2 Dietary AGEs and insulin sensitivity

The effects of reducing dietary AGE intake on insulin sensitivity are inconsistent between studies. While some report increased insulin sensitivity after a low AGE diet[76-79], others found no effect[79-81]. These discrepancies likely result from several methodological limitations. All of the abovementioned studies modulated AGEs in intervention diets by variating cooking methods, such as steaming versus grilling. As a result, heat-sensitive micronutrients such as vitamins[82, 83] and carcinogenic compounds such as acrylamide[84] may also differ and potentially explain the changes observed after a low or high AGE diet. Additionally, in all these studies, diets were not matched for energy- and macronutrient content and insulin sensitivity was assessed with HOMA-index. Furthermore, because dietary AGEs were estimated using ELISA databases, the actual quantity of AGEs is unknown. In 2016, De Courten and coworkers partly circumvented these limitations[85]. In a double-blind crossover RCT in 20 abdominally obese adults, insulin sensitivity, assessed by the hyperinsulinemic-euglycemic clamp technique, improved after a 2-week energy- and macronutrient-matched low AGE diet. While this study was the first to quantify dietary AGEs in their intervention diets using a UPLC-MS/MS dietary AGE database[57], differences in AGE intake between groups were again achieved by employing different cooking methods. These results suggest that reducing dietary AGE intake improves insulin sensitivity, but several limitations remain. A carry-over effect in this crossover trial cannot be excluded, and the mechanisms behind the improvement of insulin sensitivity were not investigated. Importantly, the effects of dietary AGEs may have been confounded by the underlying differences in food preparation methods.

1.6.3 Beta-cell function

Assessing beta-cell function is complex. The beta-cell response to stimuli depends largely on the method used and insulin levels measured in plasma do not solely reflect beta-cell function but also insulin sensitivity and hepatic insulin clearance[86]. As such, insulin secretion should always be adjusted for insulin sensitivity and derived from c-peptide deconvolution, which is not dependent on hepatic clearance[87]. Beta-cell function can be determined from fasting blood samples, as well as from techniques that involve oral administration of a meal or intravenous administration of glucose. Intravenous techniques are considered most accurate but lack physiological components of beta-cell function, such as incretin effects, which are observed in oral techniques[88]. Beta-cell function is described classically by several "static" measures and more recently by "dynamic" measures derived from mathematical modeling[89]. The progression of these static measures in individuals with normal glucose metabolism to impaired glucose metabolism and T2DM is well described. In the insulin resistant but normoglycemic state, static measures such as basal- and glucose-stimulated insulin secretion are upregulated[90]. However, in those with prediabetes, glucose-stimulated insulin secretion is decreased[91] with further deterioration in those with T2DM[22]. Conversely, the progression of dynamic measures largely depends on the methods used, and studies therefore show conflicting results. Individuals with impaired glucose tolerance show decreased dynamic measures when measured with an oral glucose tolerance test[92] but increased dynamic measures when measured with a mixed-meal test[93].

1.6.4 Dietary AGEs and beta-cell function

Although AGEs in experimental settings directly modulate beta-cell function[47-49, 94], studies on the consequences of dietary AGEs on beta-cell function are scarce. To date, only De Courten et al investigated beta-cell function after a low and high AGE diet in humans and found no difference in plasma insulin during an intravenous glucose tolerance test (IVGTT)[85]. In contrast, rats fed a baked diet high in AGEs for 24 weeks showed decreased plasma insulin during an IVGTT compared to rats fed an unbaked diet lower in AGEs[47]. However, limitations here are that only plasma insulin was reported instead of true insulin secretion as obtained by c-peptide deconvolution. Especially for the human study where a difference in insulin sensitivity was observed between groups, plasma insulin concentration can be confounded by hepatic insulin clearance[95]. Furthermore, insulin secretion in both studies was not adjusted for insulin sensitivity, so it remained uncertain whether dietary AGEs had an isolated effect on beta-cell function. As such, the effect of dietary AGEs on beta-cell function in humans warrants further investigation.

1.6.5 Macrovascular function

The arterial system serves an interrelated conduit and cushioning function as it transports blood while reducing cardiac oscillations in blood flow. As a result, it ensures constant flow in the microvascular bed of target organs[96]. With ageing, structural changes in the vascular wall, such as loss of elastin and increased collagen disposition, lead to arterial stiffening[96], a process that is accelerated in T2DM[97]. Although aortic stiffness measured as carotid-to-femoral pulse wave velocity (cfPWV) is considered the gold standard for determining arterial stiffness, carotid stiffness should also be assessed as stiffening of the carotid and aortic wall does not occur in parallel when cardiovascular risk factors are present[98]. Via several mechanisms, including left ventricular wall hypertrophy, and carotid stenosis and plaque formation[99], aortic and carotid stiffness independently predict cardiovascular events[100-102]. Furthermore, stiff arteries show reduced cushioning and the subsequent increase in blood flow oscillations may damage target-organs[103], especially those in which the microvasculature is characterized by low impedance, such as the kidney[104], eye[105], and brain[106] (Figure 1.5). Overall, the morbidity and mortality resulting from arterial stiffness[100-102] underscores the importance of identifying further risk factors.

1.6.6 Microvascular function

The microcirculation encompasses 98% of all blood vessels[107] and based on anatomical criteria includes vessels with a diameter less than 150 µm, comprising arterioles, capillaries, and venules[108]. Based on physiological criteria, small arteries with a diameter more than 150 µm that show a myogenic reduction in lumen size in respond to increasing blood pressure are also included[109]. The microcirculation serves two key functions: regulation of tissue perfusion in response to changing metabolic demands, and blood pressure regulation through modulation of vascular resistance[110]. In fact, the greatest drop in blood pressure occurs in the microvascular bed. The endothelium, the vasculature's inner lining, is central in these functions.

Regarding development of T2DM, microvascular regulation of tissue perfusion is particularly interesting as it is a major contributor to both insulin sensitivity and secretion. Under resting conditions, only one-third to one-half of the muscle capillary bed is perfused[111]. To ensure ample exchange of glucose and insulin with skeletal muscle tissue in the hyperglycemic state, insulin activates both vasodilator and vasoconstrictor pathways, of which the vasodilator pathway dominates in the healthy state[112]. More specifically, insulin stimulates eNOS, which leads to production of the potent vasodilator NO. As a result, precapillary arterioles dilate and downstream perfusion ensues. Simultaneously, this process stimulates vasomotion,

rhythmic oscillations in arteriolar diameter which regulate blood flow distribution to downstream capillaries[113]. As a result, previously hypoperfused capillaries are recruited[17], and insulin-mediated muscle glucose uptake is increased by 40%[114]. In addition to its role in insulin sensitivity, microvascular function also determines insulin production. Several experimental studies have shown that pancreatic islet endothelial function, which in turn depends mainly on adequate perfusion, is essential for normal glucose-induced insulin secretion[115, 116]. In line with this, impaired microvascular function is associated with increased incidence of T2DM in several large studies[117-119].

Regarding the development of CVD, microvascular blood pressure regulation is also interesting. Although classically changes in microvascular structure and function are regarded a consequence of hypertension[109], there is increasing evidence that microvascular changes may precede and contribute to hypertension and CVD. Retinal arteriole narrowing and venule widening are both associated with increased risk of hypertension[120, 121]. Furthermore, retinal arteriole narrowing is associated with increased incidence of coronary artery disease[122], stroke[123], and mortality due to cardiovascular events[124]. Thus, protecting microvascular function is essential in preventing T2DM and CVD.

Although microvascular function is similar throughout the circulation, endothelial genotype and phenotype depends on its location within the (micro)vascular tree[125, 126]. In line with this, endothelial function measured in larger resistance arteries and even in different microvascular beds are not necessarily the same[127-129]. As such, several microvascular measurements should be combined to draw conclusions on generalized microvascular function.

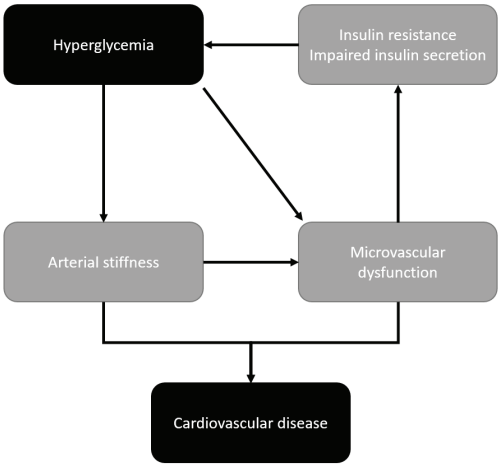


Figure 1.5 Overview of the vicious cycle between microvascular dysfunction, arterial stiffness, and hyperglycemia, ultimately leading to cardiovascular disease. Adapted from Stehouwer[9].

1.6.7 Dietary AGEs and vascular function

Although endogenous formation of AGEs may directly lead to arterial stiffness, a similar role for dietary AGEs is less expected. In line with this, there was no association between CML intake and cfPWV in 85 CVD- and complication free T2DM patients[130]. Furthermore, in a RCT by the same group, there was no difference in cfPWV in 62 prediabetes individuals after a 24-week low AGE diet[131]. This indeed suggests that dietary AGEs do not contribute to arterial stiffness. However, both studies employed ELISA-based dietary AGE databases and did not measure carotid stiffness. To fully rule out an effect of dietary AGEs on arterial stiffness, these findings need to be replicated using more sophisticated methods.

Due to its contribution to insulin sensitivity, beta-cell function, and CVD, effects of dietary AGEs on microvascular function deserve investigation. Most studies so far only assessed the consequences of dietary AGEs on plasma biomarkers of endothelial dysfunction. In a meta-analysis combining 5 studies and 174 participants, a low AGE diet significantly reduced sVCAM-1[14]. However, other plasma biomarkers, such as sICAM-1, eSelectin, and von Willebrand factor are seldomly reported. Importantly, to our knowledge, there is only one study in which effects of a low or high AGE diet on in vivo microvascular function has been determined: Semba et al found no difference in skin reactive hyperemia in 24 healthy individuals after an isocaloric low or high AGE diet for 6 weeks[132]. As such, it is currently unknown if dietary AGEs affect generalized microvascular function.

1.7 Potential underlying biological mechanisms of dietary AGEs

1.7.1 The liver, inflammation, and lipid profile

Potentially, biological effects of dietary AGEs may partly be explained by their accumulation in the liver. In line with the dramatic increase in the worldwide prevalence of obesity, the prevalence of excessive intrahepatic lipid accumulation (hepatic steatosis) has increased as well[133]. Hepatic steatosis may eventually lead to hepatic inflammation (steatohepatitis), fibrosis, and ultimately cirrhosis in what is collectively called non-alcoholic fatty liver disease (NAFLD)[134]. NAFLD is associated with insulin resistance, low-grade inflammation, and atherogenic dyslipidemia, clinical manifestations that have also been linked to a high AGE diet[14]. Although there are several other potential causes that contribute to development of NAFLD, accumulation of AGEs in the liver seems involved. Individuals with NAFLD show increased intrahepatic AGE accumulation, and this in turn correlates with the degree of steatohepatitis[135]. As dietary AGEs were previously shown to accumulate in liver of mice[64], the increase in hepatic AGEs and steatohepatitis in individuals with NAFLD

may partly be explained by intake of dietary AGEs. However, several important research gaps are present. It is unknown whether the accumulation of dietary AGEs in the liver is reversible, and if this also applies to the associated biological effects. Furthermore, the effects of dietary AGEs on intra-hepatic lipid accumulation and NAFLD severity in humans has not yet been assessed.

1.7.2 Gut microbiota

An interplay with gut microbes may also contribute to the biological effects associated with dietary AGEs. Gut microbes ferment indigestible substrates such as fibers into short-chain fatty acids (SCFAs), and proteins into branched chain amino acids (BCAAs)[136], which are associated with decreased incidence of obesity[137], insulin resistance[138], and T2DM[139]. It is well known that foods and dietary patterns may influence gut microbiota content[140]. As it is estimated that only 10% of dietary AGEs are absorbed into the circulation[62], the majority reaches the colon undigested and can potentially alter gut microbiota composition.

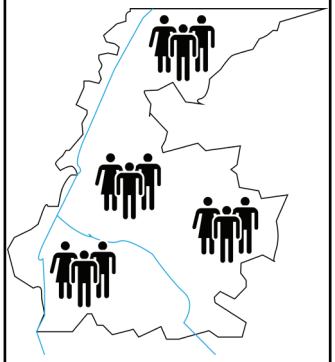
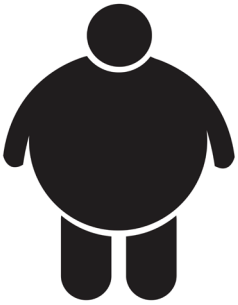
Indeed, several in vitro and animal studies show changed gut microbiota composition and SCFA production following exposure to dietary AGEs[141]. However, discrepancies are present as substrates for AGEs between studies differ. Effects of dietary AGEs on gut microbiota content in humans has been assessed in two RCTs: in both adolescents[142] as in patients on peritoneal dialysis[143], a diet low or high in AGEs based on cooking methods changed gut microbiota composition. However, it is unknown whether this explains biological effects of dietary AGEs, as results in these groups cannot be extrapolated to the general population. Alternatively, gut microbes may metabolize dietary AGEs. Recovery of dietary CML in feces is estimated at 20-30%, while recovery in plasma is estimated at 10%[62], suggesting that it is at least partly metabolized[67]. Indeed, certain bacterial strains may break down CML in vitro, producing several metabolites with unknown properties in the process[66, 144]. Thus, the interaction between dietary AGEs and the gut microbiome deserves further investigation.

1.8 Thesis outline

First, we investigated the biological effects of a diet high in AGEs and its potential reversibility in mice. To this end, we studied the effects of a baked chow diet high in AGEs, and a subsequent switch to a normal chow diet, on levels of AGEs in plasma and organs, inflammatory markers, and gut microbiota composition (Chapter 2). Next, we investigated whether intake of AGEs from the habitual diet is associated with glucose metabolism and vascular function in humans. To this end, we studied the cross-sectional associations of habitual AGE intake and arterial stiffness,

generalized microvascular function, and glucose metabolism in the large population-based cohort of The Maastricht Study (Chapter 3, 4, and 5). Finally, we determined whether modulation of dietary AGE intake influences glucose metabolism and vascular function and leads to biological effects in humans. To this end, we studied the consequences of a specifically-designed diet low and high in dietary AGEs on insulin sensitivity, beta-cell function, micro- and macrovascular function, plasma biomarkers of inflammation, lipid profile, and gut microbiota composition in humans in a double-blind RCT (Chapter 6 and 7). Findings are summarized and reflected upon in Chapter 8.

<table>
<tr><th colspan="3">THESIS OUTLINE</th></tr>
<tr><td colspan="3">Chapter 1 - General introduction

Glucose metabolism, vascular function, and dietary AGEs</td></tr>
<tr><td>Mouse intervention trial</td><td>Observational cohort</td><td>Human intervention trial</td></tr>
<tr><td>  </td><td>  </td><td>  </td></tr>
<tr><td>Chapter 2
Reversible effects of a baked chow diet high in AGEs on AGEs in plasma and organs, gut microbiota composition, and inflammation</td><td>Chapter 3
Associations of dietary AGEs and arterial stiffness

Chapter 4
Associations of dietary AGEs and generalized microvascular function

Chapter 5
Associations of dietary AGEs and glucose metabolism</td><td>Chapter 6
Effects of a diet low and high in AGEs on insulin sensitivity, insulin clearance, beta-cell function, vascular function, AGEs, and inflammation

Chapter 7
Effects of a diet low and high in AGEs on gut microbiota composition</td></tr>
<tr><td colspan="3">Chapter 8 - General discussion

Findings, implications, strengths, limitations, and conclusions</td></tr>
</table>

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dietary AGEs in DUTCH SPICED CAKE
CML: 0.8; CEL 1.2; MG-H1: 20.3 mg/100g

Chapter 2

Dietary advanced glycation endproducts (AGEs) increase their concentration in plasma and tissues, result in inflammation, and modulate gut microbial composition in mice; evidence for reversibility

Katja C.W. van Dongen[1], Armand M.A. Linkens[1], Suzan M.W. Wetzels, Kristiaan Wouters, Tim Vanmierlo, Marjo P.H. van de Waarenburg, Jean L.J.M. Scheijen, Willem M. de Vos, Clara Belzer[2], Casper G. Schalkwijk[2]

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[1] and [2] equal contribution

Abstract

Background: Dietary advanced glycation endproducts (AGEs) are associated with negative biological effects, possibly due to accumulation in plasma and tissues and through modulation of inflammation and gut microbiota.

Objectives: We investigated biological consequences of a baked chow diet high in AGEs on their levels in plasma and organs, inflammation, and gut microbiota composition and determined whether these consequences are reversible.

Methods: Young healthy C57BL/6 mice were fed a standard chow (*n* = 10) or a baked chow high AGE-diet (*n* = 10) (~1.8–6.9 fold increased protein-bound N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl) lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1)) for 10 weeks or a switch diet with baked chow for 5 weeks followed by 5 weeks of standard chow (*n* = 10). We assessed accumulation of AGEs in plasma, kidney, and liver and measured inflammatory markers and gut microbial composition.

Results: After 10 weeks of baked chow, a substantial panel of AGEs were increased in plasma, liver, and kidney. These increases were normalized after the switch diet. The inflammatory z-score increased after the baked chow diet. Gut microbial composition differed significantly between groups, with enriched *Dubosiella* spp. dominating these alterations.

Conclusions: A high AGE-diet led to an increase of AGEs in plasma, kidney, and liver and to more inflammation and modification of the gut microbiota. These effects were reversed or discontinued by a diet lower in AGEs.

Introduction

Advanced glycation endproducts (AGEs) are abundantly present in processed food products and may contribute to beneficial effects on flavor, smell, and shelf life[1]. These dietary AGEs are formed during the non-enzymatic reaction between free amino acids and reducing sugars, via the so-called Maillard reaction[2]. There is increasing evidence in humans and animals that consumption of dietary AGEs contribute to AGEs measured in plasma[3, 4] and organs[5] and that a diet high in dietary AGEs in humans has negative biological effects, such as low-grade inflammation, endothelial dysfunction, and insulin resistance[6]. However, it is currently unknown whether AGE accumulation in tissues and the negative biological effects associated with a high AGE diet are reversible. Furthermore, how dietary AGEs are involved in the aforementioned biological effects remains poorly understood.

Dietary AGEs may exert their biological effects, at least partly, through modulation of the gut microbiota composition, which in turn is increasingly recognized to play a fundamental role in the pathophysiology of obesity, diabetes, and cardiovascular disease[7]. Although there are some discrepancies, several animal studies have shown that a heat-treated diet high in dietary AGEs can modulate gut microbiota composition[8-12]. However, the effects on inflammatory markers or increases in protein-bound and free AGE levels in plasma and/or tissues were not addressed in all of these studies. Furthermore, it is currently unknown whether changes in gut microbiota composition following a high AGE diet are reversible.

In light of the above, we hypothesized that mice fed a high dietary AGE diet for 10 weeks show increased blood and tissue AGEs, increased inflammatory markers, and different microbiota composition as compared to mice fed a standard dietary AGE diet. In addition, we studied whether changes following the high dietary AGE diet were reversible by implementing a switch after 5-weeks of high dietary AGE diet to the standard dietary AGE diet for 5 subsequent weeks.

Material and Methods

Animal studies

To obtain a high AGE diet, a standard rodent chow (ssniff, Soest, Germany) was baked at 160 °C for two hours (hereafter referred to as “baked chow”). 9-week-old female C57BL/6/OlaHsd mice were randomly divided in three cages in groups of 10 (Figure 2.1). After a one-week acclimatization period, the mice were divided in three groups fed either standard chow (*n* = 10), baked chow high in dietary AGEs (*n* = 10), or a

"switch diet" (*n* = 10) for 10 weeks. In the switch diet group, mice were first fed the baked chow diet for 5 weeks, and subsequently the standard chow diet for 5 weeks. All diets were provided ad-libitum. Fecal and blood samples were collected after the 1-week acclimatization period. Blood samples were collected after 5 weeks of dietary intervention, while fecal samples were collected every 2.5 weeks. At the end of the 10-week dietary intervention, all mice were sacrificed by anesthetic overdose and plasma, liver and kidneys, and feces (from the rectum) were collected. To test whether accumulation of AGEs in organs after a high dietary AGE diet are reversible, two extra groups of 10 mice were allocated to either 5 weeks of the standard chow diet or 5 weeks of the baked chow diet under the same conditions as mentioned above. At the end of the 5-week dietary intervention, all mice were sacrificed. All experiments were approved by the local ethical committee for animal experiments of Hasselt University and performed according to institutional guidelines (matrix 201503).

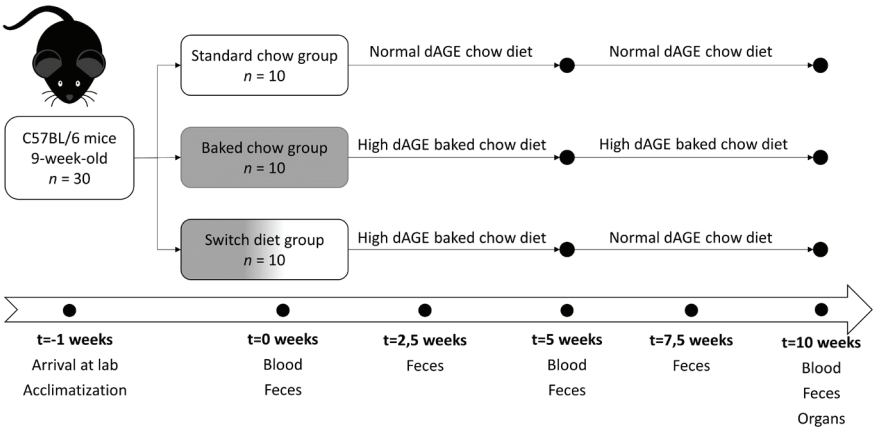

Figure 2.1 Design of the main study. dAGE: dietary AGE.

Advanced glycation end products and oxoaldehyde measurements

AGEs were measured in chow diets, plasma, liver, and kidney. AGEs in plasma were measured as this reflects uptake of dietary AGEs from the gastrointestinal tract. We chose to measure AGEs in liver and kidney as these are major organs and are highly susceptible to AGE accumulation and AGE-induced damage[13, 14]. Livers and kidneys were homogenized using a Mini-bead beater homogenizer (Biospec) and 250 µL sodium phosphate buffer (0.1 M) supplemented with protease inhibitor (Roche) and 0.02% Triton-x (Sigma- Aldrich).

Free- and protein-bound AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}- (1-carboxyethyl) lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon- 2-yl)-ornithine (MG-H1) were

analyzed in plasma, liver, and kidney homogenates by LC-MS/MS after extraction as described in detail before[15, 16]. Additionally, free-, protein-bound AGEs, and the oxoaldehydes methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3DG) were analyzed in pulverized animal diets as described previously[15, 17].

Inflammation markers

IFN-γ, IL-10, IL-6, KC/GRO, and TNF-α were measured in plasma using MSD V-PLEX multiplex assay platforms (Meso Scale Diagnostics, Rockville, MD, U.S.A.). C-reactive protein (CRP) was measured using mouse CRP ELISA DuoSet kit (R&D system, Minneapolis, Minn, U.S.A.). For IFN-γ and IL-6, some samples were below the limit of detection (LOD) (<0.12 pg/ml for IFN-γ, n = 3, <3 pg/ml for IL-6, n = 14). These missing values were substituted by half the LOD, a commonly used method[18] (thus 0.06 pg/ml for IFN-γ and 1.5 pg/ml for IL-6).

Microbial 16S rRNA sequencing

DNA was isolated from the fecal pellets using a bead-beating procedure in combination with the customized MaxWell® 16 Tissue LEV Total RNA Purification Kit (XAS1220; Promega Biotech AB, Stockholm, Sweden) as described before[19]. Triplicate PCR reactions of the 16S ribosomal RNA (rRNA) gene V4 region (515-F GTGYCAGCMGCCGCGGTAA, 806-R GGACTACNVGGGTWTCTAAT; 10 µM each) were applied to the template DNA isolates (20 ng/µL) with a unique barcoded sequences library approach to identify individual fecal pellets with a total volume of 35 µL per reaction. Formed PCR products were qualitatively confirmed and purified as described before[19]. 200 ng of each sample was pooled after quantification with the Qubit® dsDNA BR Assay Kit and subsequently sequenced (Illumina NovaSeq 6000, paired-end, 150 bp; Eurofins Genomics Europe Sequencing GmbH, Konstanz, Germany). The 16S rRNA gene sequencing raw data has been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB41378.

Microbiota data processing and analysis

Sequences of the 16S rRNA gene were analyzed using the NG-Tax 2.0 pipeline20 with default settings, which generates de novo exact match sequence clusters: amplicon sequence variants (ASVs). ASVs with a relative abundance below 0.1% were removed and the threshold for taxonomic assignment was set at 80%. The SILVA 16S rRNA gene reference database release 13221 was used to assign taxonomy. The program

R (version 3.6.1) was used for further data analysis. The Phyloseq package[22] (version 1.30.0) was applied to combine the ASV table with the phylogenetic tree and the metadata. ASVs with a relative abundance > 0.1% in one of the individual samples were included for further data analysis. Bray-Curtis dissimilarities (beta diversity) were assessed with the Phyloseq package. Relative abundance composition plots at phylum and genus level were created using the Microbiome package[23]. The web-based tool Linear Discriminant Analysis (LDA) Effect Size (LEfSe)[24] was used to identify differential abundant taxa between the diet groups per sampling week. Spearman's rank correlations of microbial compositional data, glomerated at genus level, with relevant clinical data were performed with the associate function of the Microbiome package[23] (version 1.8.0). Therefore, subsets of each sampling week were created and taxa with a relative abundance > 1% in one of the samples were included.

Statistical analysis

Analyses were conducted using SPSS version 25 for Windows (IBM Corporation, Armonk, NY, USA). AGE and oxoaldehyde content of standard and baked chow are presented as mean ± SD, and comparisons of AGE and oxoaldehyde content of standard and baked chow diets were tested using the two-tailed unpaired Students T-test. Comparisons between free and protein-bound AGEs in plasma and tissues and inflammatory markers between groups were performed using the Mann-Whitney-U test. Free and protein-bound AGEs in plasma and tissues and inflammatory markers are presented as median [IQR] due to skewed distributions. To increase statistical efficiency and reduce multiple testing when correlating inflammatory markers to the microbiota data, an inflammatory z-score was calculated by combining TNF-α, IFN-γ, KC/GRO, IL-6 and the inverse of IL-10 (1/IL-10) as described previously[25]. To this end, first, z-scores for all individual parameters were calculated as follows: (individual value minus whole study population mean value)/ whole study population SD, thus resulting in a standardized variable ranging from approximately - 2.5 to + 2.5 SD with a mean of 0. Second, as these individual z-scores share the same unit, they were averaged, resulting in one single inflammation score, which was subsequently standardized. Permutational multivariate analysis of variance (PERMANOVA) of microbial beta diversity was assessed by application of the Adonis function (999 permutations) of Vegan package[26] (version 2.5–6). LEfSe analysis revealed differential abundant taxa if the *p* value of the non-parametric Kruskal-Wallis test between two diet groups was < 0.05 and the effect size of the logarithmic LDA score > 2.0. *p* values of correlations of microbial taxa with clinical data were adjusted for multiple testing using the Benjamini & Hochberg false discovery rate (FDR). All analysis were considered statistically significant with *p* values < 0.05 unless otherwise stated.

Results

Increased levels of AGEs in chow after baking

To assess the effect of baking on chow diets, we compared free and protein-bound AGEs, and oxoaldehyde content between standard chow and baked chow. While levels of free CEL and MG-H1 increased after the baking procedure (34%, $p < 0.05$, and 266%, $p < 0.01$, respectively), free CML decreased after the baking procedure (-54%, $p < 0.05$) (Figure 2.2). In contrast, levels of protein-bound AGEs in chow were uniformly and significantly increased following the baking procedure: 299% for protein-bound CML ($p < 0.001$), 691% for CEL ($p < 0.001$) and 182% for MG-H1 ($p < 0.001$) (Figure 2.2). Likewise, levels of oxoaldehydes were also significantly increased following the baking procedure: 108% for MGO ($p < 0.05$), 53% for GO ($p < 0.05$) and 379% for 3DG ($p < 0.001$) (Figure 2.2).

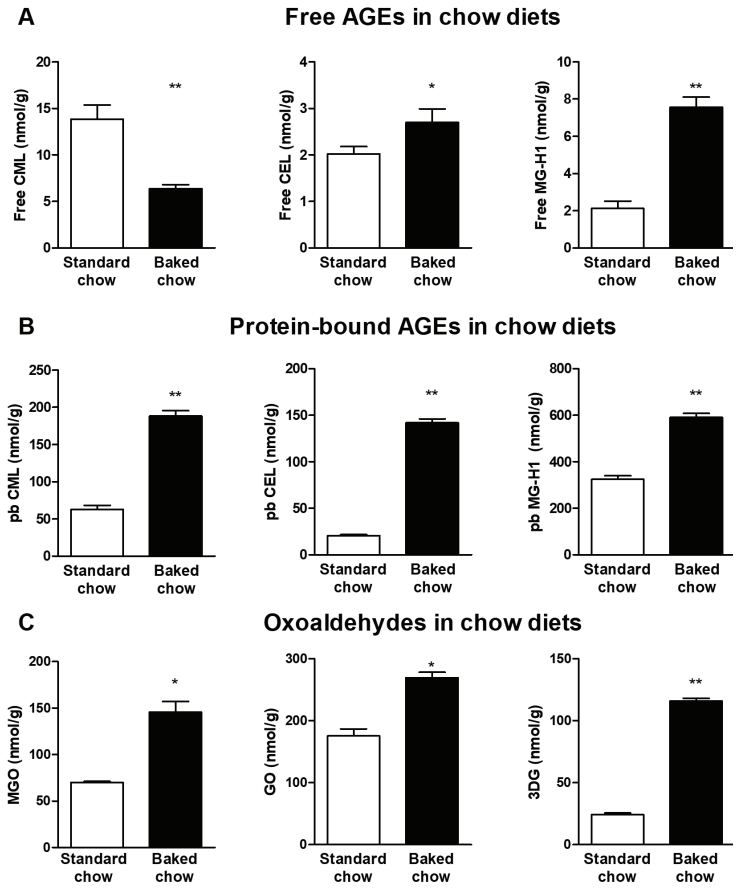

Figure 2.2 Free AGEs (A), protein-bound AGEs (B), and oxoaldehydes (C) in standard chow and baked chow, as analyzed by UPLC-MS/MS. CML: N^{ε}-(carboxymethyl) lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. MGO: methylglyoxal. GO: glyoxal. 3DG: 3-deoxyglucosone. pb: protein-bound. For free and protein-bound AGEs, bars and error bars indicate mean and SD of 5 samples per diet. For oxoaldehydes, bars and error bars indicate mean and SD of 2 samples per diet. * indicates $p < 0.05$

Mice fed baked chow ate less than mice fed standard chow, but weight gain was similar

To test whether weight gain over 10 weeks in young mice was different after the consumption of a baked chow diet, standard chow diet, or the switch diet, we determined food intake and weight at baseline and after 10 weeks. Mice fed the baked chow diet for 10 weeks ate less than mice fed the standard chow diet or switch diet: 2.96 g/day, 3.77 g/day, and 3.13 g/day, respectively (Table 2.1). In line with this, body weight was higher after 10 weeks of standard chow compared to baked chow or the switch diet, with median (g) [IQR] of 23.4 [22.2;24.1], 21.1 [20.5;21.2], and 21.8 [20.3;22.5], respectively. However, body weight of mice fed the baked chow diet was already lower at baseline as compared to mice fed the standard chow diet, thus percentage weight gain was not different between mice fed standard chow, baked chow, or the switch diet after 10 weeks, with median (%) [IQR] of 22 [19;29], 19 [15;22], and 17 [16;23], respectively (Table 2.1).

Table 2.1 Mice characteristics

	Standard Chow	Baked Chow	Switch Diet	*P* standard vs baked	*P* standard vs switch	*P* Baked vs switch
Weight baseline (g)	19.1 [17.9-19.5]	18.0 [17.1-18.2]	18.1 [16.4-19.3]	**0.019**	0.165	0.481
Weight sacrifice (g)	23.4 [22.2-24.1]	21.1 [20.5-21.2]	21.8 [20.3-22.5]	**0.001**	**0.004**	0.481
Weight gain (g)	4.2 [3.5-5.2]	3.3 [2.8-3.8]	3.1 [2.9-4.1]	**0.043**	**0.035**	0.796
Weight gain (%)	22 [19-27]	19 [15-22]	17 [16-23]	0.218	0.123	1.000
Food intake (g/day)	3.77	2.96	3.13			

Sample size *n* = 10 per group. Weight is presented as median [IQR]. Food intake was measured for each group as whole, thus statistical differences between groups cannot be computed. Significant differences ($p < 0.05$) are shown bold.

A baked chow diet increases both free and protein-bound AGEs in plasma

To assess the impact of the chow diets on the level of AGEs after 10 weeks, we compared free and protein-bound AGEs in plasma between groups. All free AGEs in plasma were higher after baked chow compared to standard chow, with median (nmol/L) [IQR] of 536 [406;795] vs. 355 [332;430], $p = 0.004$ for CML, 228 [129;374] vs. 118 [86;135], $p = 0.011$, for CEL, and 242 [89;303] vs. 76 [61;102], $p = 0.019$ for MG-H1 (Figure 2.3A). Results for protein-bound AGEs in plasma were similar, except for MG-H1. After 10 weeks of the baked chow diet, protein-bound CML and CEL in plasma were higher compared to the standard chow diet, with median (nmol/L) [IQR] of 853 [805;919] vs. 561 [542;599], $p < 0.001$, and 311 [227;345] vs. 207 [183;235] $p = 0.011$, respectively. Protein-bound MG-H1 in plasma was lower after baked chow compared to standard chow: 1464 [1377;1506] vs. 1630 [1502;1911], $p = 0.011$ (Figure 2.3B).

A baked chow diet increases AGEs in liver and kidneys, but mainly in the free form

We next determined the difference in AGE levels in organs after 10 weeks of the baked chow or standard chow diet. In liver, free CML and MG-H1, but not CEL, were higher after baked chow compared to standard chow, with median (nmol/g of protein) [IQR] of 8.0 [7.2;8.5] vs. 5.4 [4.9;5.7] for CML ($p < 0.001$), 2.3 [1.6;3.3] vs. 0.8 [0.8;0.9] for MG-H1 ($p < 0.001$), and 8.0 [7.4;8.7] vs. 8.1 [6.8;9.6] for CEL ($p = 0.730$) (Figure 2.3C). Contrarily, protein-bound AGEs in liver were not different, although there was a trend for increased protein-bound CML in liver after baked chow compared to standard chow: 21 [20;22] vs. 18 [17;21] for CML ($p = 0.063$), 44 [39;46] vs. 41 [37;44] for CEL ($p = 0.393$), and 24 [21;28] vs. 25 [23;33] for MG-H1 ($p = 0.436$) (Figure 2.3D). In kidney, free AGEs were higher after baked chow compared to standard chow, although the difference in free CML did not reach statistical significance: 224 [143;284] vs. 161 [119;179] for CML ($p = 0.052$), 114 [67;147] vs. 48 [37;57] for CEL ($p < 0.001$), and 17 [8;26] vs. 5 [5;6] for MG-H1 ($p = 0.001$) (Figure 2.3E). In contrast, protein-bound CML in kidney, but not CEL or MG-H1, was higher in kidney after baked chow compared to standard chow: 26 [24;29] vs. 22 [19;24] for CML ($p < 0.001$), 19 [18;22] vs. 21 [17;22] for CEL ($p = 0.796$), and 28 [22;44] vs. 31 [25;36] for MG-H1 ($p = 0.971$) (Figure 2.3F).

The accumulation of AGEs in plasma and organs after a baked chow diet is reversible

We next studied whether the accumulation of AGEs after a baked chow diet can be reversed by a standard chow diet. We first assessed whether AGEs in plasma and organs were already increased after 5 weeks of the baked chow diet. In general, all free and protein-bound AGEs were increased in plasma, kidney, and liver of mice fed the baked chow diet for 5 weeks compared to mice fed the standard chow diet for 5 weeks (Supplementary Figure 2.1). However, protein-bound MG-H1 in plasma was not significantly different between mice fed the baked chow diet for 5 weeks compared to the standard chow diet, with median (nmol/g of protein) [IQR] of 1599 [1487;2128] vs. 1484 [1371;1704]. In addition, free CEL was lower and protein-bound MG-H1 was not different in liver of mice fed the baked chow diet compared to the standard chow diet: 8.3 [8.0;8.9] vs. 9.6 [8.8;10.4] , $p = 0.007$ and 25.3 [21.5;28.9] vs. 26.5 [24.9;30.8], $p = 0.280$, respectively. The accumulation of AGEs in plasma, kidney and liver after 5 weeks of a baked chow diet were reversible, as most AGEs in plasma, liver, and kidney were significantly decreased after the switch to standard chow (Figure 2.3A-F). Remarkably, free CML, free CEL, and protein-bound CEL in kidney were even lower after the switch diet as compared to 10 weeks of standard chow, with median (nmol/g of protein) [IQR] of 96 [85;102] vs. 161 [119;179], $p = 0.011$, 31 [29;33] vs. 48 [37;57], $p = 0.015$, and 16 [15;19] vs. 21 [17;22], $p = 0.009$, respectively (Figure 2.3E and 3F).

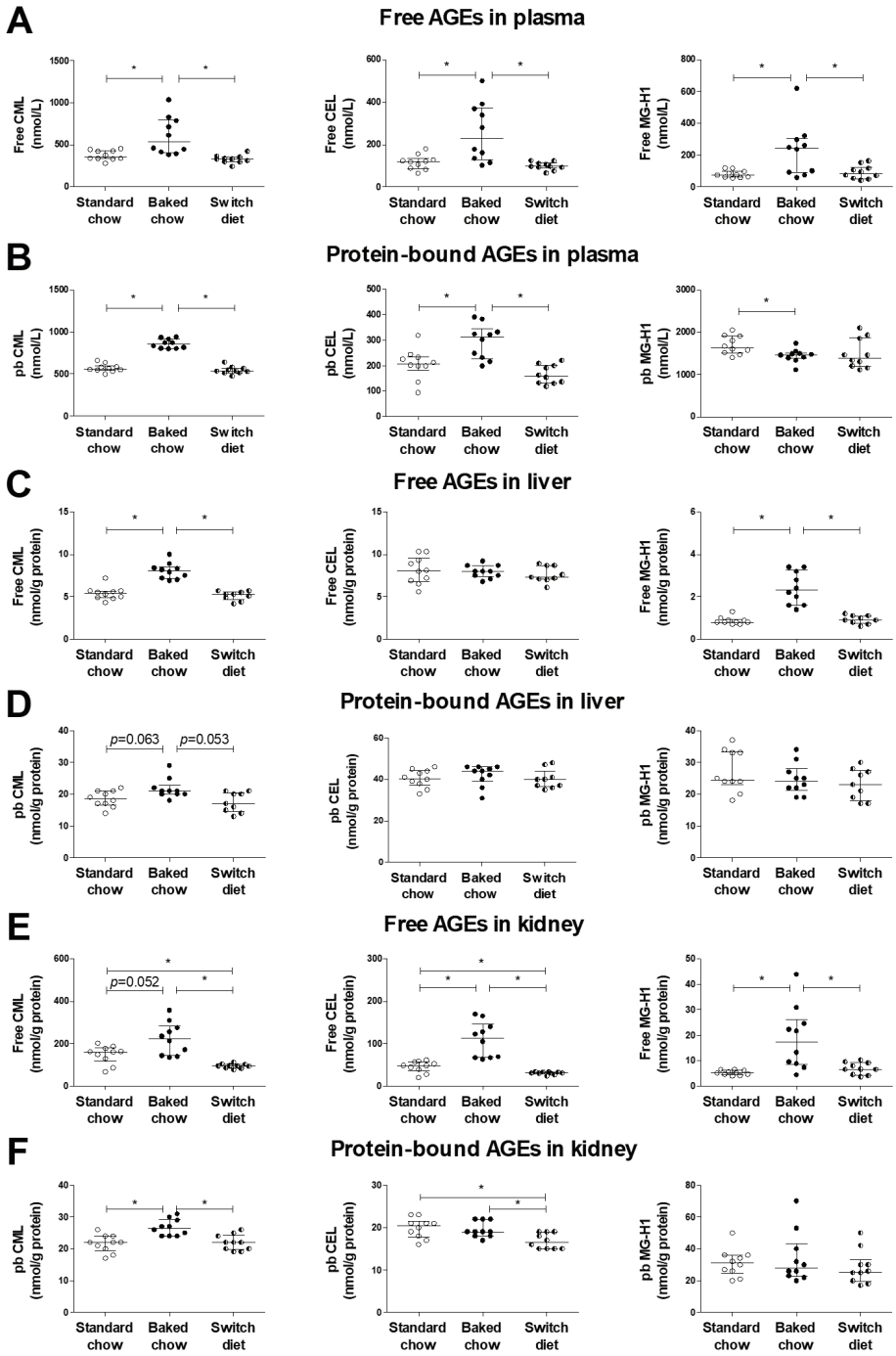

Figure 2.3 Free and protein-bound AGEs in plasma (A,B), liver (C,D), and kidney (E,F) of mice after 10 weeks of standard chow, baked chow, and the switch diet, as analyzed by UPLC-MS/MS. CML: N^{ε}-(carboxymethyl)lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. pb: protein-bound. Center bars of scatter dots indicate median values while whiskers indicate inter quartile range. * indicates $p < 0.05$ for the difference between chow diets. $N = 10$ for all groups, except for free and protein-bound AGEs in liver for the switch diet group, where $n = 9$.

A baked chow diet affects some markers of inflammation

To assess whether inflammation is increased after a baked chow diet compared to a standard chow diet, a panel of inflammatory markers were analyzed individually. TNF-α, IFN-γ, KC/GRO, CRP, and IL-6 were not different after 10 weeks of baked chow or standard chow (Figure 2.4A). However, the anti-inflammatory cytokine IL-10 was significantly lower after baked chow compared to standard chow: 9.4 pg/ml [8.5;10.4] vs. 12.8 pg/ml [10.1;14.8], $p = 0.011$ (Figure 2.4A). We next combined the inflammatory markers into an overall inflammatory z-score that also included IL-10. The inflammatory z-score was higher after 10 weeks of baked chow than after 10 weeks of standard chow, with median (SD) [IQR] of 0.37 [-0.13;1.31] vs. - 0.39 [-1.05;0.20], $p = 0.029$ (Figure 2.4B). Then we determined whether the increase in inflammation after a baked chow diet could be reversed by a standard chow diet. We first assessed whether inflammatory markers in plasma were already increased after 5 weeks of the baked chow diet. In contrast to AGEs in plasma and organs, inflammatory markers in plasma and the inflammatory z-score were not already increased after 5 weeks of baked chow compared to 5 weeks of standard chow (Supplementary Figure 2.2). In fact, plasma TNFα was lower after 5 weeks of baked chow compared to 5 weeks of standard chow, with median (pg/ml) [IQR] of 5.85 [5.16–6.39] vs. 7.50 [6.48;8.42], $p = 0.017$. Although inflammatory markers in plasma were not already increased after 5 weeks of baked chow, the subsequent increase in inflammatory markers after 10 weeks of baked chow could be prevented by the switch to standard chow, with inflammatory z-score median [IQR] 0.37 [-0.13;1.31] vs- 0.41 [-0.81;0.26], $p = 0.034$ (Figure 2.4B).

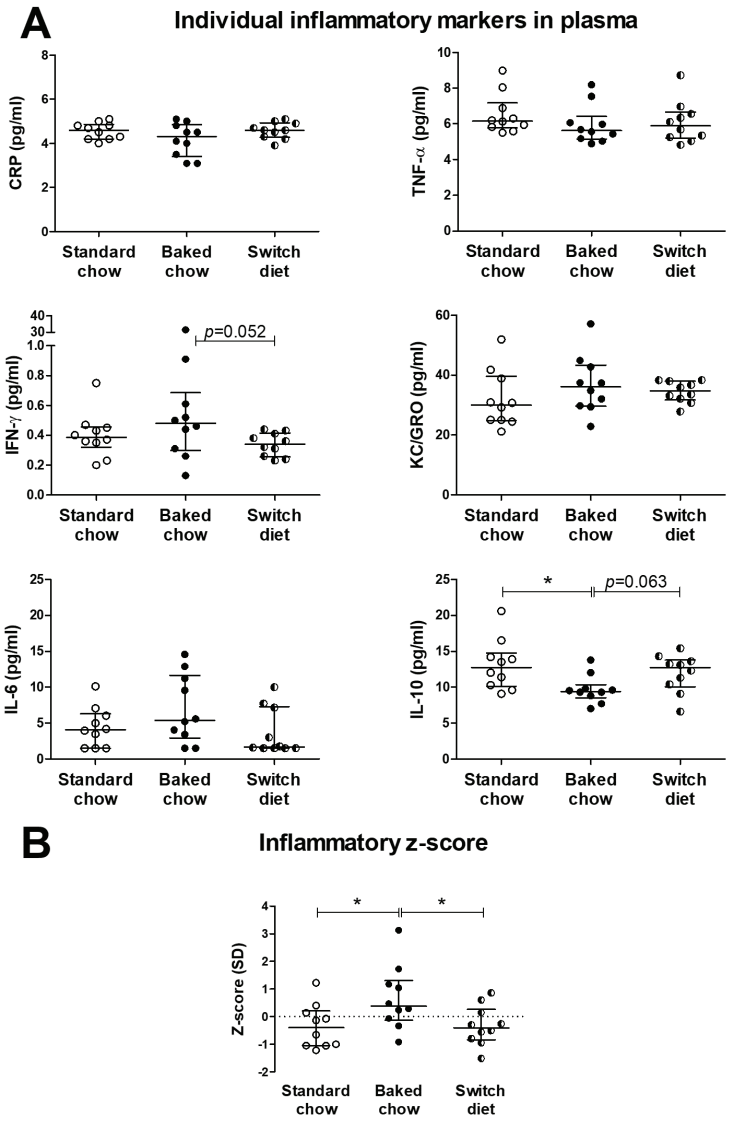

Figure 2.4 Inflammatory mediators in plasma of mice after 10 weeks of standard chow, baked chow, and the switch diet. A: individual inflammatory mediators. B: Inflammatory z-score consisting of TNF-α, IFN-γ, KC/GRO, IL-6, and IL-10. Center bars of scatter dots indicate median values while whiskers indicate inter quartile range. * indicates $p < 0.05$ for the difference between chow diets. $N = 10$ for all groups.

Gut microbial composition is altered after feeding a baked chow diet, and is reversible

Based on Bray-Curtis beta diversity dissimilarity distances, differences in overall microbial composition were shown in principal coordinate analysis (PCoA) plots from week 7.5 onwards (Figure 2.5). Where in week 5 almost all samples were still

distributed over the four quadrants (Axis 1 = 23.2%, Axis 2 = 13.5%), in week 7.5 the fecal samples from the mice fed the baked chow diet almost all clustered together in the bottom left quadrant, while almost all fecal samples from the standard chow diet clustered together in the upper right quadrant (Axis 1 = 23.6%, Axis 2 = 12%). The fecal samples from the mice fed the switch diet were distributed over the two upper quadrants and were located in between the samples of the baked chow and the standard chow diet. In week 10 the fecal samples of the mice fed the baked chow and the standard chow diet again were distributed over opposite quadrants, while the samples of the mice fed the switch diet were located in between these two diet groups, as expected (Axis 1 = 26.2%, Axis 2 = 23%). It should be noted that in week 10 not all fecal pellets were collected for each group (n = 6 for the baked chow group; n = 4 for the standard chow group; n = 2 for the switch group). Further PERMANOVA analysis revealed that the provided diet explained a statistically significant (p < 0.001) variation in microbial composition from week 5 onwards with 19.5% variance explained by the diet in week 5, 22.9% in week 7.5 and 35.2% in week 10. Bray-Curtis dissimilarities of week 0 and week 2.5 were not statistically significant. Their corresponding PCoA plots are shown in the supplementary materials (Supplementary Figure 2.3).

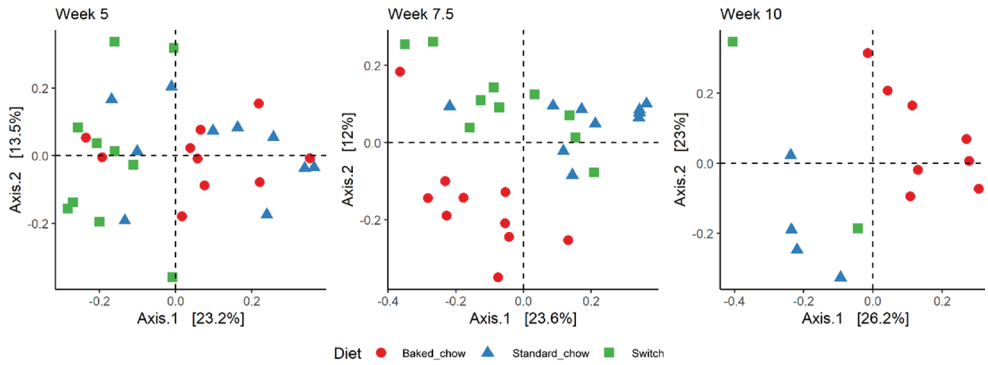

Figure 2.5 Principal coordinate analysis plots (PCoA) of Bray-Curtis' beta diversity dissimilarities of the fecal pellets per week. Each data point represents one fecal pellet of an individual mouse and labelled per diet (red circles for baked chow diet; blue triangles for the standard chow diet; green squares for the switch diet).

Relative abundance composition plots both on phylum level and genus level per sampling week showed differences between the different diet groups (Supplementary Figure 2.4A and B), especially at genus level. In order to identify taxa which contributed to observed differences in microbial composition between the diet groups as assessed with Bray-Curtis dissimilarities, LEfSe analysis of the microbiota composition was performed per sampling week from week 5 onwards. Differential abundant taxa were identified after comparing the microbial composition of the baked chow with the standard chow (Figure 2.6) and the switch with the standard

chow (Supplementary Figure 2.5), and were visualized in cladograms (circular phylogenetic trees). Comparing the baked chow with the standard chow revealed that at week 5, six genera (i.e. *Olsenella*, *Ruminococcaceae_UCG_009*, *Dubosiella*, *Turicibacter*, *Parasutterella* and *Akkermansia* with respective average relative abundances (%) of 0.7, 0.1, 3.6, 0.5 and 3.2) and four families (i.e. *Atopobiaceae, Erysipelotrichaceae, Burkholderiaceae, Akkermansiaceae*) were enriched after the baked chow diet and three genera (i. e. *Clostridiales_vadinBB60_group_uncultured, Roseburia and Faecalibaculum* with respective average relative abundances (%) of 0.1, 4.5 and 1.0) and one family (i.e. *Clostridiales_vadinBB60_group*) was enriched after consumption of standard chow. At week 7.5, twelve genera (i.e. *Bifidobacterium, Rikenella, Eubacterium_xylanophilium_group, Lachnoclostridium, Roseburia, Ruminiclostridium_5, Ruminococcaceae_uncultured, Ruminococcaceae_UCG_009, Ruminococcaceae_UCG_014, Dubosiella, Faecalibaculum,* and *Desulfovibrio* with respective average relative abundances (%) of 0.2, 0.3, 0.1, 0.6, 1.5, 0.3, 1.1, 0.1, 0.3, 13.1, 0.2 and 1.7) and three families (i.e. *Bifidobacteriaceae, Erysipelotrichaceae* and *Desulfovibrionaceae*) were assigned as enriched taxa for the baked chow group, while for the standard chow diet five genera (i.e. *Bacteroides, Muribaculaeae_uncultured, Muribaculum, Lactobacillus* and *Marvinbryantia* with respective average relative abundances (%) of 1.2, 46.5, 1.2, 10.5 and 0.7) and four families (i.e. *Bacteroidaceae, Muribaculaceae, Lactobacillaea* and *Clostridiales_vadinBB60_group*) were enriched. The analysis performed at week 10 identified four genera (i.e. *Clostridium_sensu_stricto_1, Dubosiella, Turicibacter* and *Parasutterella* with respective average relative abundances (%) of 0.6, 26.2, 0.9 and 0.7) and three families (i.e. *Clostridiaceae_1, Erysipelotrichaceae* and *Burkholderiaceae*) to be enriched for the baked chow diet, while for the standard chow diet five genera (i.e. *Bacteroides, Lactobacillus, Lachnospiraceae_UCG_004, Oscillibacter* and *Ruminiclostridium* with respective average relative abundances (%) of 1.2, 22.5, 0.1, 0.4 and 0.7) and two families (i.e. *Bacteroidaceae* and *Lactobacillaceae*) were identified to be enriched.

The microbiota of baked chow-fed mice was found to be consistently enriched in the genus *Dubosiella*, while that of mice fed the standard chow diet were enriched in *Lactobacillus* and *Bacteroides* from week 7.5 onwards. The highest number of identified taxa after LEfSe analysis were found in week 7.5 which could be explained by the fact that in week 10 not all fecal pellets were collected for each group (n = 6 for the baked chow group; n = 4 for the chow group; n = 2 for the switch group), while for week 7.5 fecal pellets from all mice (n = 10 per group) were collected and subsequently analyzed for its microbial composition.

LEfSe analysis of the switch diet with the standard chow diet (Supplementary Figure 2.5) resulted in the highest number of enriched taxa in week 5 which decreased

to week 7.5 and further to week 10. In week 10 only three genera (i.e. *Dubosiella, Turicibacter* and *Ileibacterium* with respective average relative abundances (%) of 2.9, 3.7 and 12.4) were shown to be higher abundant in the switch diet. This indicates that the microbial composition as affected by the baked chow diet is a diet-dependent reversible change.

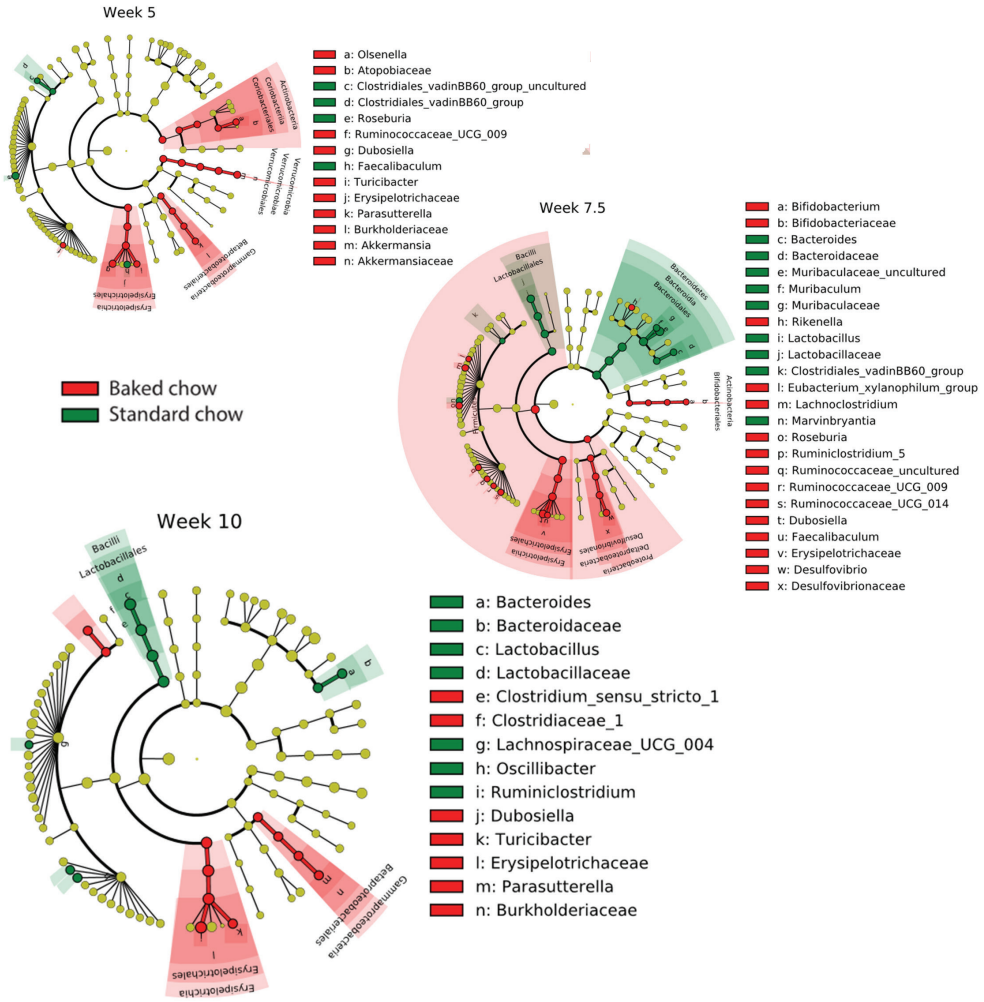

Figure 2.6 LEfSe results of the significant different taxa found by comparing the mice with the baked chow diet (red) to the standard chow diet (green), sampled in week 5 (n = 10 per group), 7.5 (n = 10 per group) and week 10 (baked chow: n = 6; standard chow: n = 4). Nomenclature was based on the highest achievable taxonomic resolution level. The alpha value was set to 0.05 and the log10 LDA score threshold to 2.0.

Gut microbiota composition data are associated with clinical parameters

To assess whether gut microbiota composition was associated with other measured outcomes (i.e. AGE plasma and tissue levels, inflammatory markers), Spearman's

rank correlations were determined after 5 and 10 weeks for mice fed the baked chow and the standard chow diet (Figure 2.7). In week 5, the genera *Olsenella* and *Turicibacter* showed a statistically significant positive correlation with free plasma CML levels (*Olsenella*: ρ = 0.769, *p* = 0.008; *Turicibacter*: ρ = 0.686, *p* = 0.030) and CEL (*Olsenella*: ρ = 0.711, *p* = 0.024; *Turicibacter*: ρ = 0.671, *p* = 0.031). In week 10, no bacterial taxa were statistically significantly correlated with the other outcomes after correction for multiple testing, possibly due to less fecal pellets being collected at week 10. However, in week 10 several taxa showed a trend towards a high positive or negative correlation (ρ > 0.7 or ρ < -0.7) with these outcomes. As such, *Dubosiella* showed a high positive correlation with free kidney CEL levels (ρ = 0.719), L*achnospiraceae NK4A136 group* with CRP levels in plasma (ρ = 0.709), *Alloprevotella* with protein-bound MG-H1 in plasma (ρ = 0.755), *Rikenellaceae RC9 gut group* with protein-bound MG-H1 levels in the kidney (ρ = 0.745) and *Alistipes* with plasma CRP levels (ρ = 0.709). On the other hand, *Rikenellaceae RC9 gut group* showed a high negative correlation with free CML in the liver (ρ = -0.729), *Muribaculaceae uncultured bacterium* with free CML and CEL in plasma (ρ = -0.797 and -0.825), protein-bound CML in plasma (ρ = -0.755), free CML in the liver (ρ = -0.753), and free CML and MG-H1 in the kidney (ρ = -0.748 and ρ 0.783). *Muribaculum* showed high negative correlations with free CML and CEL in plasma, and free MG-H1 in plasma and kidney (ρ = -0.797, -0.825, -0.718, and -0.734, respectively).

Since not all fecal pellets were collected at week 10, an additional Spearman's rank correlation analysis was performed using the microbial composition data of week 7.5 with other measured outcomes determined at week 10 (Supplementary Figure 2.6). This revealed multiple statistically significant correlations. *Dubosiella* showed a positive correlation with free MG-H1 in liver (ρ = 0.754, *p* = 0.018), free CML in liver (ρ = 0.683, *p* = 0.078), protein-bound CML in plasma (ρ = 0.796, *p* = 0.014) and free CEL in kidney (ρ = 0.771, *p* = 0.014). *Desulfovibrio* showed a positive correlation with free MG-H1 levels in the liver (ρ = 0.667, *p* = 0.098). On the other hand, *Muribaculum* showed a negative correlation with free MG-H1 both in liver (ρ = -0.777, *p* = 0.014) and kidney (ρ = -0.715, *p* = 0.040). *Marvinbryantia* showed a negative correlation with protein-bound CEL in plasma (ρ = -0.744, *p* = 0.021). Although statistically significant results differed between Spearman's rank correlations using microbiome composition data from week 7.5 or week 10, the overall trend in found correlations was comparable.

Bacteroides and *Lactobacillus* showed a time-dependent increase from week 5 to week 10 towards negative correlations with free and protein-bound AGE levels in plasma and tissues, while *Dubosiella* showed a time-dependent increase towards positive correlations with free and protein-bound AGE levels in plasma and tissues. These results are in line with the taxa identified in the LEfSe analysis.

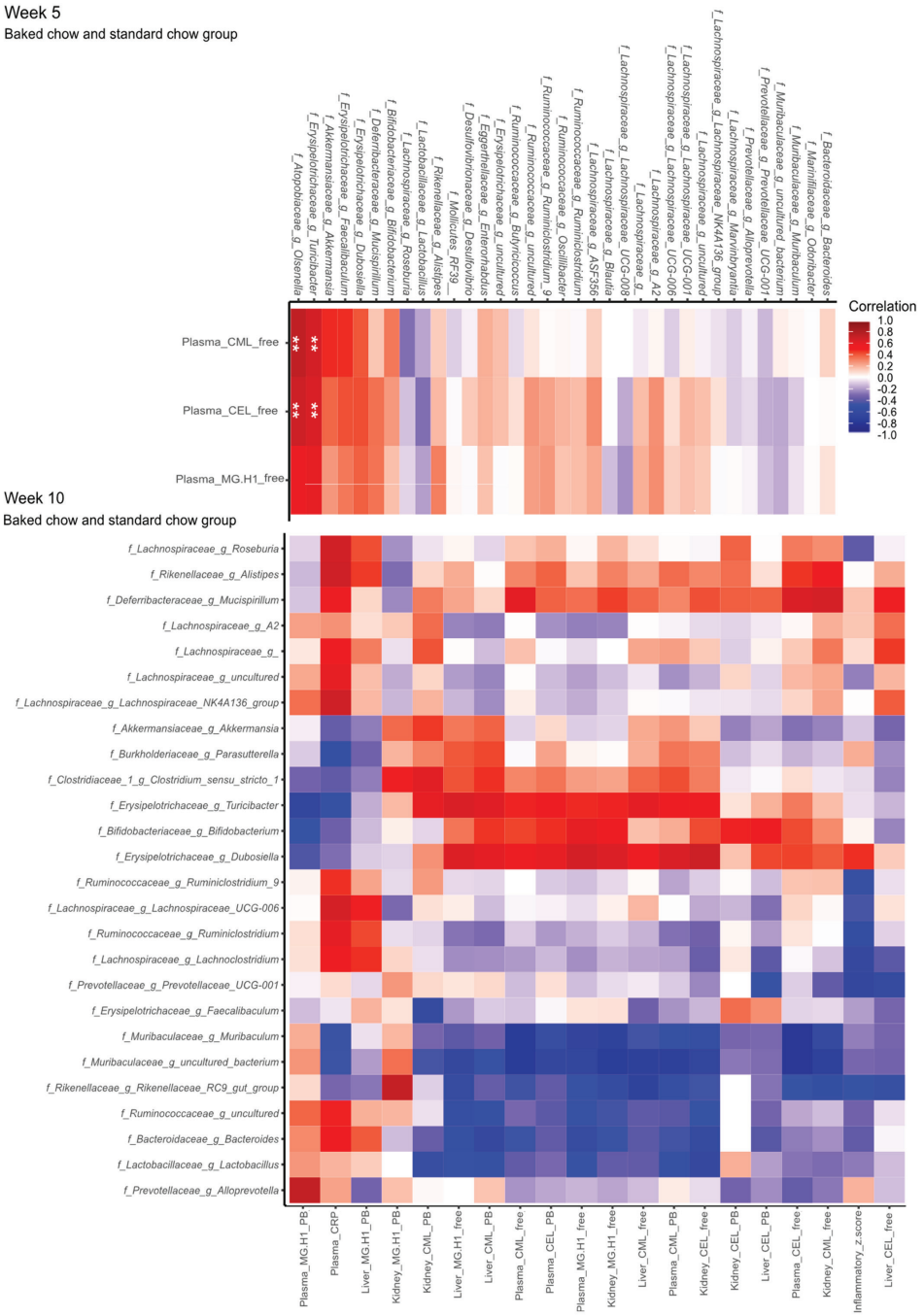

Figure 2.7 Heatmaps of Spearman's rank correlation coefficient of relative abundance microbial composition data with relevant clinical parameters per sampling week, of mice in the baked chow and standard chow group. Bacterial taxa with a relative abundance >1% in one of the samples were included. Statistically significant correlations after adjustment for multiple testing are marked with * ($p < 0.1$) or ** ($p < 0.05$). Nomenclature was based on the highest achievable taxonomic resolution level. pb: protein-bound.

Discussion

Here, we show that consumption of a baked diet containing high levels of AGEs leads to an increase of AGEs in plasma, kidney, and liver and to a reduction of circulating IL-10 in addition to changes in gut microbiota composition in young mice. These effects were reversible or discontinued by switching the high AGE diet to a diet with lower levels of AGEs. AGEs are generated in foods during various preparation methods involving dry heat, such as baking, grilling, frying, and toasting. Indeed, we saw a marked increase of the AGEs CML, CEL and MG-H1 after the baking of chow diet at 160° Celsius for two hours. Although food intake was lower in mice receiving the baked chow diet in comparison to the standard chow diet, total AGE intake was still increased in mice fed the baked chow diet. In line with this, free CML, CEL, and MG-H1 in plasma were higher in mice receiving the baked chow diet compared to mice receiving the standard chow diet. Although both free (CEL and MG-H1) and protein-bound (CML, CEL, and MG-H1) AGEs were increased in chow diets after baking, the uptake of dietary AGEs into the circulation is likely to depend mainly on digestion and absorption of protein-bound AGEs. It was previously shown that absorption of AGEs in vitro takes place mainly in the form of dipeptides, instead of free amino acid AGEs[27]. In addition, levels of protein-bound AGEs in baked chow were approximately 30–80 fold higher than levels of free AGEs. In line with this, the increase in free plasma CML after the baked chow diet occurred in absence of an increase in free CML in baked chow.

Surprisingly, protein-bound CML and CEL in plasma were also higher, while protein-bound MG-H1 was lower in mice receiving the baked chow diet compared to mice receiving the standard chow diet. We did not expect a direct increase in plasma protein-bound AGEs following the high AGE baked chow diet. This would be in line with our previous data in humans showing that the free form of AGEs in plasma, but not the protein-bound form, is associated with dietary AGE intake in participants of the CODAM study[3]. However, randomized controlled trials (RCTs) in humans are still inconclusive as there are reports of both an increase in protein-bound AGEs in plasma[28] and no change[29] after a high AGE diet. This distinction is important, as the origin of both forms of AGEs is different, and it has implications for their biological effects, for example as free AGEs do not have affinity for the receptor for AGEs (RAGE)[30-32].

We also observed some surprising findings in organs of mice fed the baked chow diet. While we observed higher levels of most free AGEs in liver and kidney in these mice, we also observed higher protein-bound CML in kidney. Direct evidence for dietary AGE accumulation in organs is already available in animal models, but free-

and protein-bound AGEs have not been assessed simultaneously[5, 33-35]. Tessier et al. used ^{13}C-labeled CML to discriminate between dietary and endogenous CML and found that dietary CML accumulated in several organs of mice, but they could not discriminate between free and protein-bound CML due to low quantities of tissue being available[5]. The increase in protein-bound CML and CEL in plasma and protein-bound CML in kidney of mice fed the baked chow diet in the present study could be the result from indirect effects of dietary AGEs. It has previously been shown that dietary CML and MGO-modified albumin lead to significant increases of NF-κB and RAGE in kidneys of piglets and white adipose tissue respectively[33, 36]. As such, protein-bound AGEs in plasma and tissues may reflect increased endogenous formation of AGEs, and not a direct uptake of dietary AGEs. In addition, we also measured reactive precursors of AGEs in the chow diet, which were increased upon heating. The increased levels of these reactive oxoaldehydes in baked chow diet may give rise to increased endogenous levels of protein-bound AGEs.

In line with the increase in AGEs in plasma and tissue, we observed reduced levels of circulating IL-10 in mice fed the baked chow diet as compared to mice fed the standard chow diet. Also driven by this, the inflammatory z-score was significantly higher after the baked chow diet compared to the standard chow diet. In line with our findings, Rajan et al. observed a significant increase in expression of several pro-inflammatory cytokines in 12-week old mice after a 6-month dietary intervention with baked chow diet[37] and Mastrocola et al. also observed a decrease in IL-10 after feeding a high AGE diet for 22 weeks in 4-week old mice[8]. Although with the current data we cannot provide insights in the origin of increased inflammation following the baked chow diet, the increase in protein-bound CML in kidney is suggestive of involvement of the kidneys. Future studies could provide insight in a potential direct link between increased protein-bound levels in a specific tissue with the origin of observed inflammation. Nonetheless, the increase in inflammation after the baked chow diet may provide some insight into the mechanisms behind the biological effects of dietary AGEs. Circulating IL-10 levels are lower in obese insulin resistant individuals and treatment with IL-10 improves lipid-induced insulin resistance[38]. As such, the decrease in circulating IL-10 after the baked chow diet could potentially contribute to the decrease in insulin sensitivity observed in humans after a high AGE diet[29]. However, in humans, the increase in inflammation after a high AGE diet is observed in some RCTs[39] but not all[40] and deserves further investigation.

To our knowledge, we are the first to show that the accumulation of AGEs in plasma and organs after a diet high in dietary AGEs is reversible. We showed that most AGEs in plasma, kidney, and liver were already increased after 5 weeks of the baked chow diet and that this accumulation of AGEs is fully reversible, by switching the diet to

standard chow for 5 subsequent weeks. Although reversibility may be expected for AGEs in plasma, as they are rapidly cleared by the kidneys[41], the latter does not necessarily apply to dietary AGEs accumulating in organs. We can currently only speculate on how dietary AGEs are transported into organs, if they end up in the intra- or extracellular matrix, and if glycated proteins (i.e. protein-bound AGEs) show different protein turnover rates. For example, the extracellular space is less subjected to protein turnover[42]. Additionally, glycated proteins in food show resistance to enzymatic breakdown in the gastrointestinal tract[43] and this could also potentially apply to enzymatic protein turnover. Inflammation, on the other hand, was not already increased after 5 weeks of the baked chow diet. Although mice fed the switch diet showed decreased inflammation compared to mice fed the baked chow diet for 10 weeks, we cannot speak of true reversibility. Nonetheless, dietary AGEs have been associated with negative biological effects in humans[6], but also several negative clinical outcomes, such as insulin resistance[29], weight gain[44], and vertebral fractures[45]. Therefore, the important finding of reversibility of AGE accumulation in plasma and organs may have beneficial implications for those whom ingest a diet high in AGEs to lower the dietary AGE-associated negative biological effects and clinical outcomes. Furthermore, the observation of reversible AGE accumulation after modulation of dietary AGEs also further strengthens their causal link.

Next to effects of baked chow diets on AGE levels in plasma and tissues and inflammatory markers, the baked chow diet also altered the gut microbiota composition of mice compared to the standard chow diet. From week 5 onwards significant effects of the baked chow diet on the gut microbiota composition were observed by assessing Bray-Curtis dissimilarities. Interestingly, statistically significant effects on gut microbiota composition were earlier observed (i.e. week 5) compared to effects on the inflammatory z-score and IL-10 levels in plasma (i.e. week 10). In the baked chow group, the genus *Dubosiella* was consistent enriched. *Dubosiella* was recently isolated from mice[46], and has not been associated with specific functions before. However, *Dubosiella* belongs to the family *Erysipelotrichaceae* and was found to be related to *Allobaculum stercoricanis*[46]. The genus *Allobaculum* spp. was increased in relative abundance in two animal studies after a glycated or high AGE diet[9, 47], which might imply a specific genera-cluster or family as a potential target for a high AGE diet. Interestingly, this does not corroborate with previous findings by Yang et al. who found an enrichment in the order *Erysipelotrichales* after the control diet instead of the high AGE diet. However, no specific genus was identified and it is relevant to note that old mice (15-month-old) were studied[12].

On the other hand, *Lactobacillus* and *Bacteroides* were consistent enriched in the standard chow group, corresponding to a relative decrease in the baked chow group,

which corroborates partially or fully with earlier findings showing a contraction in *Lactobacillus* spp. or *Bacteroides* spp.[8-11, 48]. However, not all animal studies found this contraction in *Lactobacillus* spp. or *Bacteroides* spp.[12], indicating the need for multiple studies to derive clear and consistent conclusions regarding effects on gut microbiota composition.

In week 5, the genera *Olsenella* and *Turicibacter* showed a positive statistically significant correlation with free CML and CEL in plasma, while in week 10 no statistically significant correlations were found after correction for multiple testing. This could be explained by the higher number of parameters involved in the correlations of week 10 compared to week 5. Overall, *Dubosiella* showed high correlations with most parameters measured at week 10, and was included in statistical significant positive correlations when applying microbiome composition data from week 7.5 instead of week 10. Our data suggests that *Dubosiella* spp. are associated with a diet high in AGE levels. However, further work is required to identify the function or role of *Dubosiella* spp. in order to explain this link with AGEs. A hypothesized explanation of the enrichment of *Dubosiella* spp. in the baked chow diet group is the utilization of AGEs as a substrate by the bacterium. Multiple bacterial strains were reported in literature to degrade CML. For example *Cloacibacillus evryvenis*[49], isolated from human feces, and *Escherichia coli* strains[50] were shown to degrade CML in vitro, which proves the ability of specific gut bacteria to utilize an AGE. An attempt was made to investigate whether the effects of the baked chow diet on gut microbial composition were reversible by including the switch diet group. The results of both the Bray-Curtis dissimilarities and LEfSe analysis indicated that the trend of changing microbiota composition due to the baked chow diet is reversible. In week 10, LEfSe analysis identified 3 genera (i.e. *Dubosiella, Turicibacter* and *Ileibacterium*) to be enriched in the switch diet group. These findings suggest that minor differences in microbiota composition between the standard chow and the switch diet were still observed, which can be explained by the fact that the gut microbiota requires time to adapt towards a new diet composition. It could be that a 5-week dietary intervention of the standard chow diet after 5 weeks of the baked chow diet was too short to completely adapt and consequently forming a comparable microbiota composition as in the 10-week standard chow diet group.

Our study has several strengths. Protein-bound and free AGEs were measured both in the chow diets as well as in plasma and organs of mice with the highly specific gold standard UPLC-MS/MS technique. To our knowledge, we are the first to concurrently measure both forms of AGEs in both the experimental diet and tissue compartments. In addition, the validity of our findings is increased by inclusion of a control group and the switch group. Also, inclusion of the switch group enables us to show that changes

in AGE accumulation following a high AGE diet are reversible in nature. Moreover, it is important to highlight that the chow baking conditions were not substantially different from the conditions used in other studies, and while AGE levels in the baked chow diet were increased, they still represented realistic dietary levels. Finally, we used very young, healthy mice without any underlying disease.

Our study also has several limitations. Primarily, we cannot rule out that the baking procedure has had effects on chow other than increasing AGEs, such as decreasing vitamin bioavailability or increasing acrylamide formation. Additionally, mice weighed less at the end of the study after the baked chow diet compared to the standard chow diet, and food intake was lower of mice in the baked chow diet group. However, percentual increase of weight did not differ between groups, and in addition bacterial taxa associated with weight loss, such as *Allobaculum*[51] or *Akkermansia muciniphila*[52], were not showing a similar trend towards lower weight in the baked chow diet group. If anything, lower food intake in the baked chow group reduced exposure to dietary AGEs and lead to underestimations of their effects. Finally, our observations of reversible AGE accumulation in kidney and liver may not be extrapolated to other organs. Likewise, our findings in mice cannot be directly extrapolated to humans, as species differences exist in for example metabolic rate and dietary habits, as also in gut microbiota composition[53].

In summary, intake of dietary AGEs results in reversible elevated levels of AGEs in plasma and organs and alterations in the gut microbiota composition with a change in the inflammatory profile of healthy young mice. Randomized controlled trials on the effects of dietary AGEs on gut microbiota in humans are needed.

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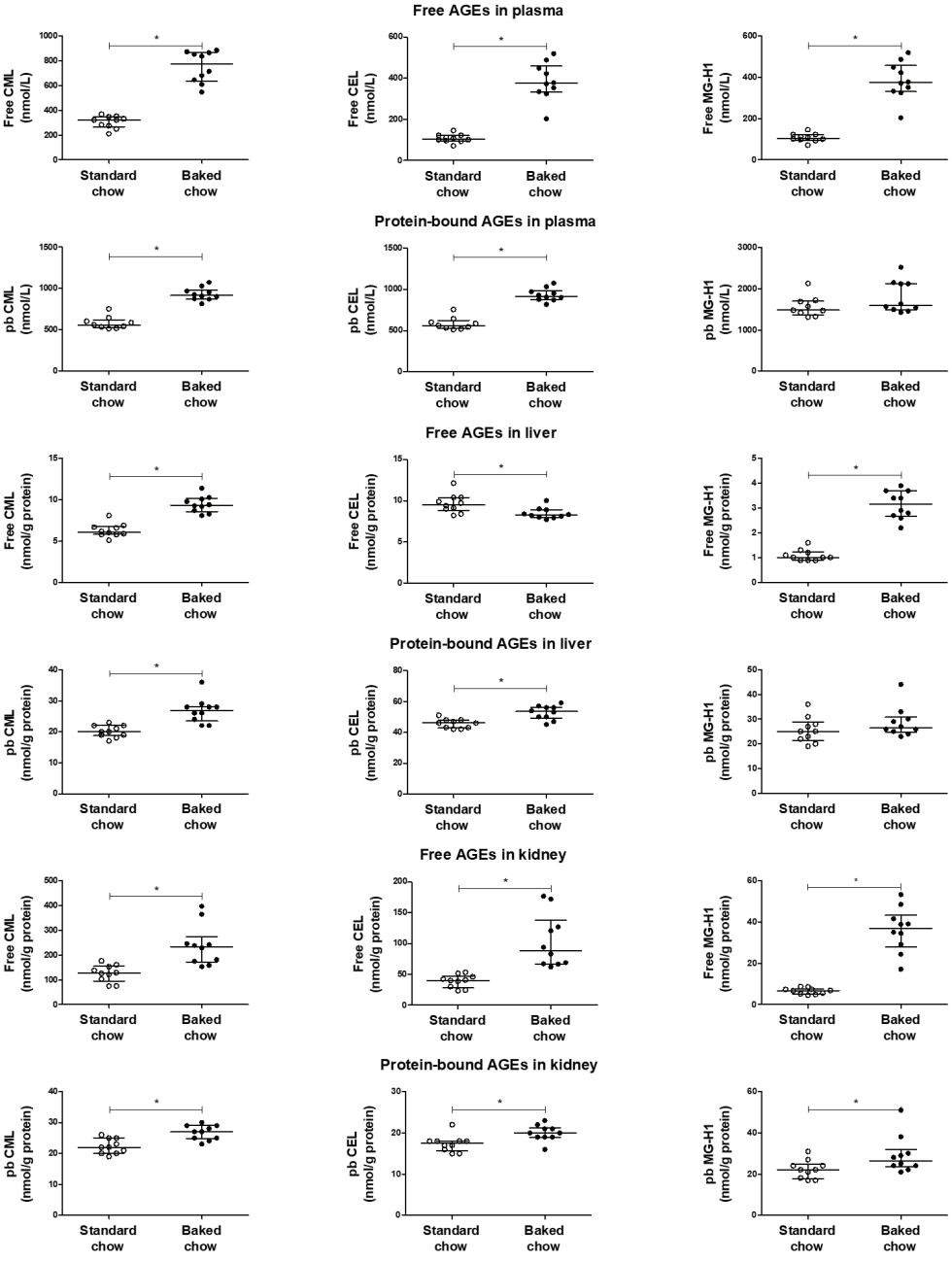
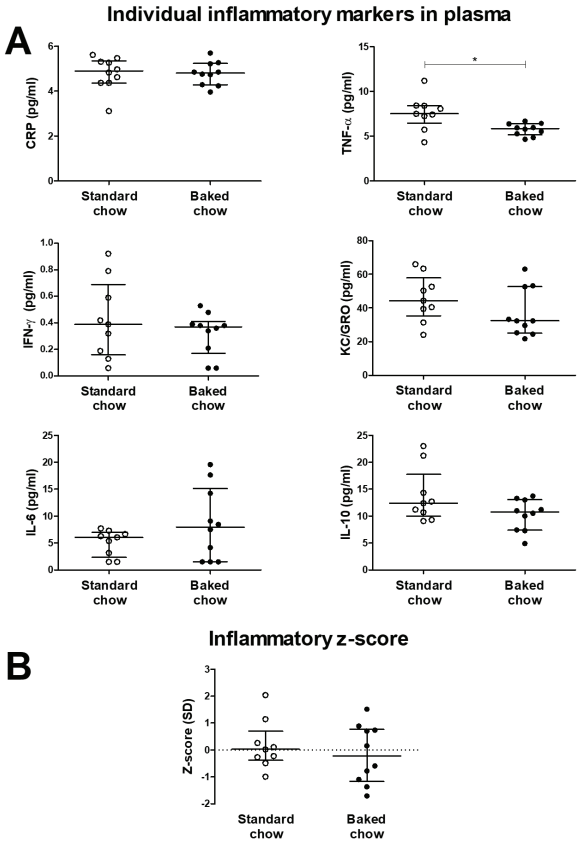
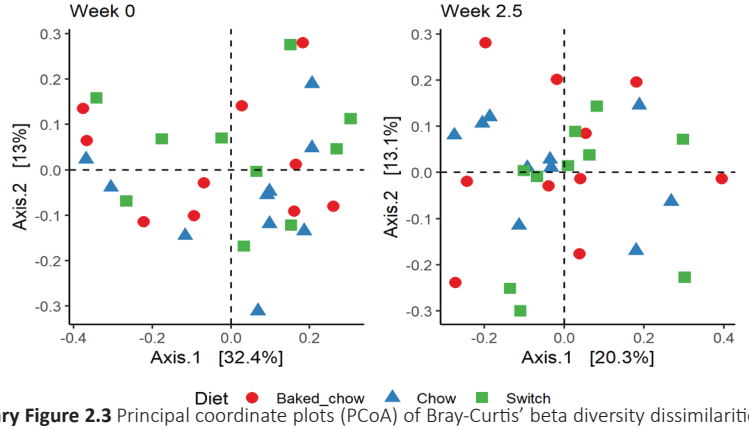
Supplementary Figures



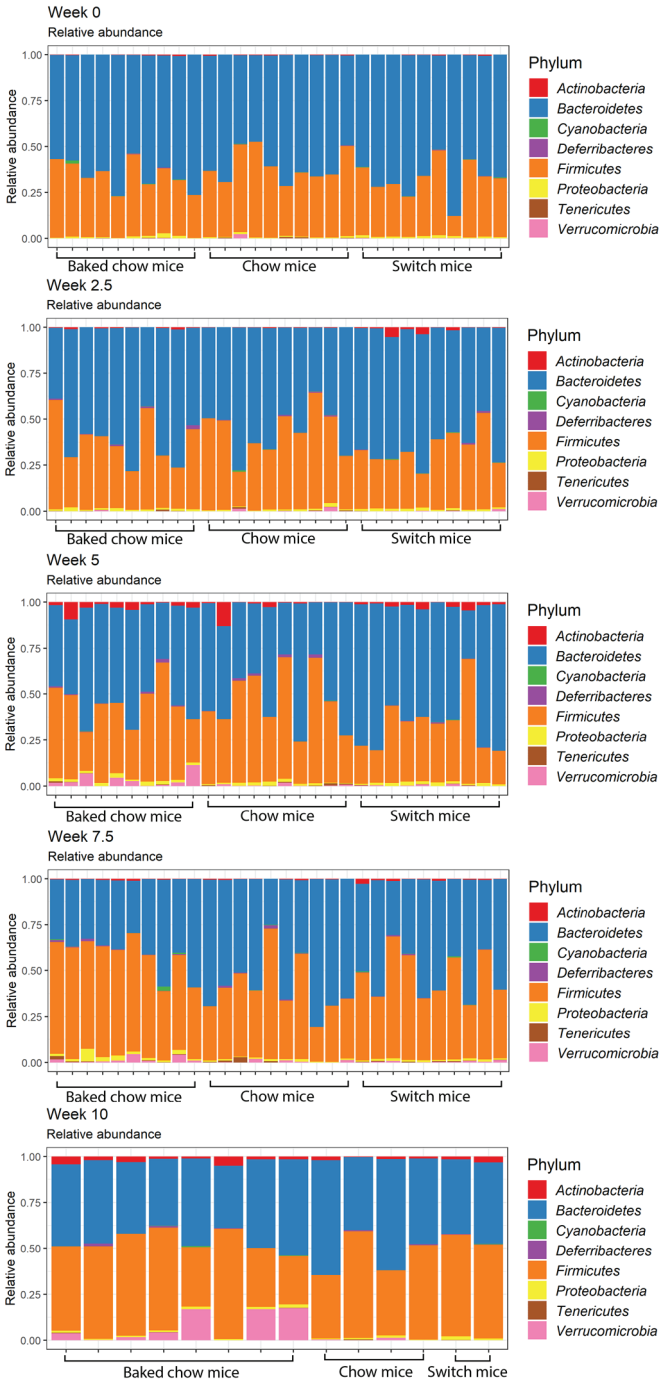
Supplementary Figure 2.1 Free and protein-bound AGEs in plasma, kidney and liver of mice after 5 weeks of the standard chow diet or baked chow diet. CML: N^{ε}-(carboxymethyl)lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. pb: protein-bound. Center bars of scatter dots indicate median values while whiskers indicate inter quartile range. * indicates $p < 0.05$ for the difference between chow diets. $n = 10$ for both groups, except for plasma protein-bound AGEs in the standard chow group, where $n = 9$.



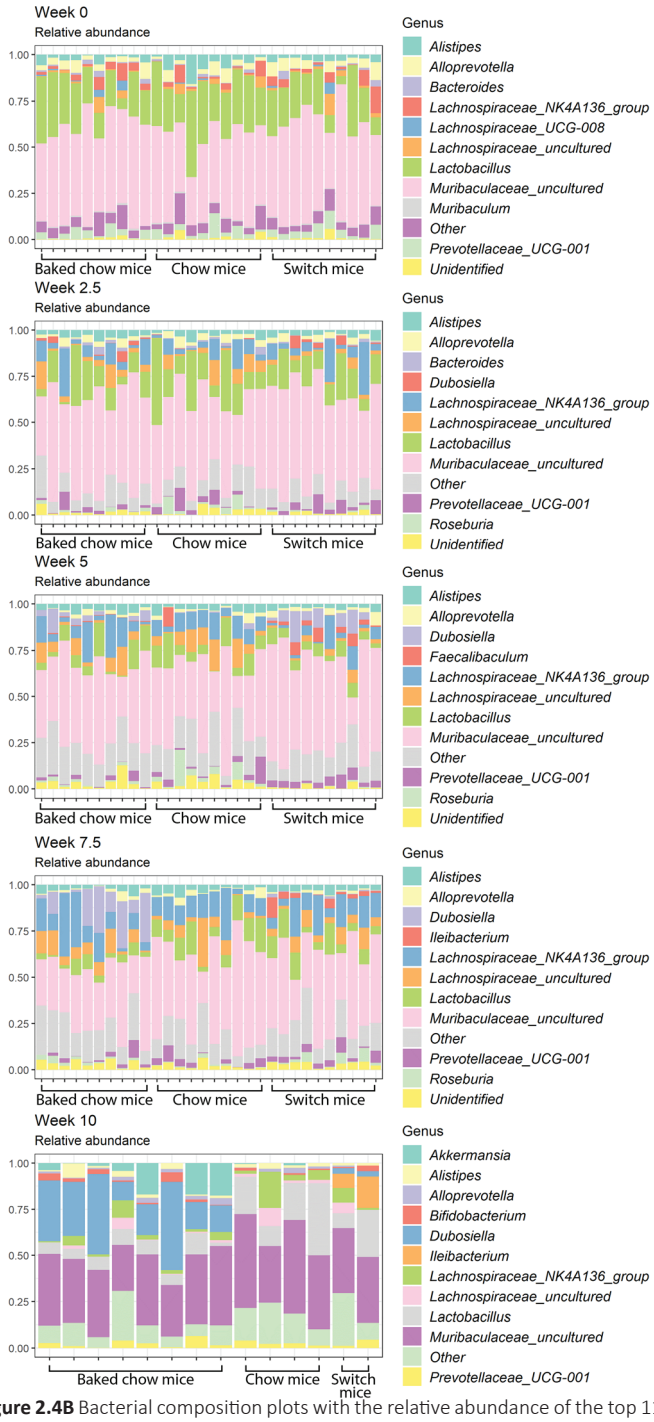
Supplementary Figure 2.2 Inflammatory mediators in plasma of mice after 5 weeks of standard chow or baked chow. A: individual inflammatory mediators. B: Inflammatory z-score consisting of TNF-α, IFN-γ, KC/GRO, IL-6, and IL-10. Center bars of scatter dots indicate median values while whiskers indicate inter quartile range. * indicates $p < 0.05$ difference between chow diets. $N = 9$ for the standard chow group, $n = 10$ for the baked chow group.



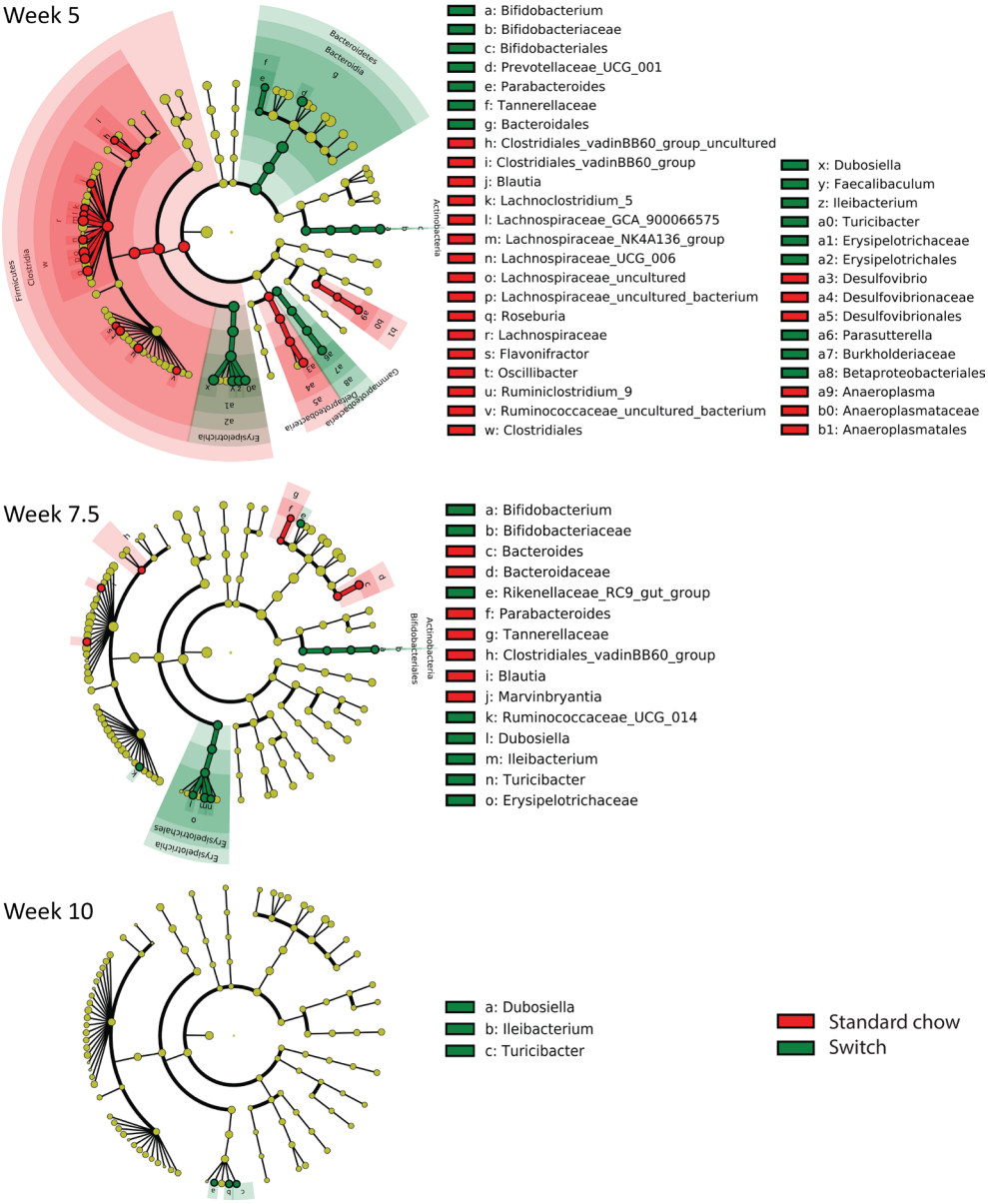
Supplementary Figure 2.3 Principal coordinate plots (PCoA) of Bray-Curtis' beta diversity dissimilarities of the fecal pellets for week 0 and week 2.5. Each data point represents one fecal pellet of an individual mouse and labelled per diet (red circles for baked chow diet; blue triangles for the standard chow diet; green squares for the switch diet).



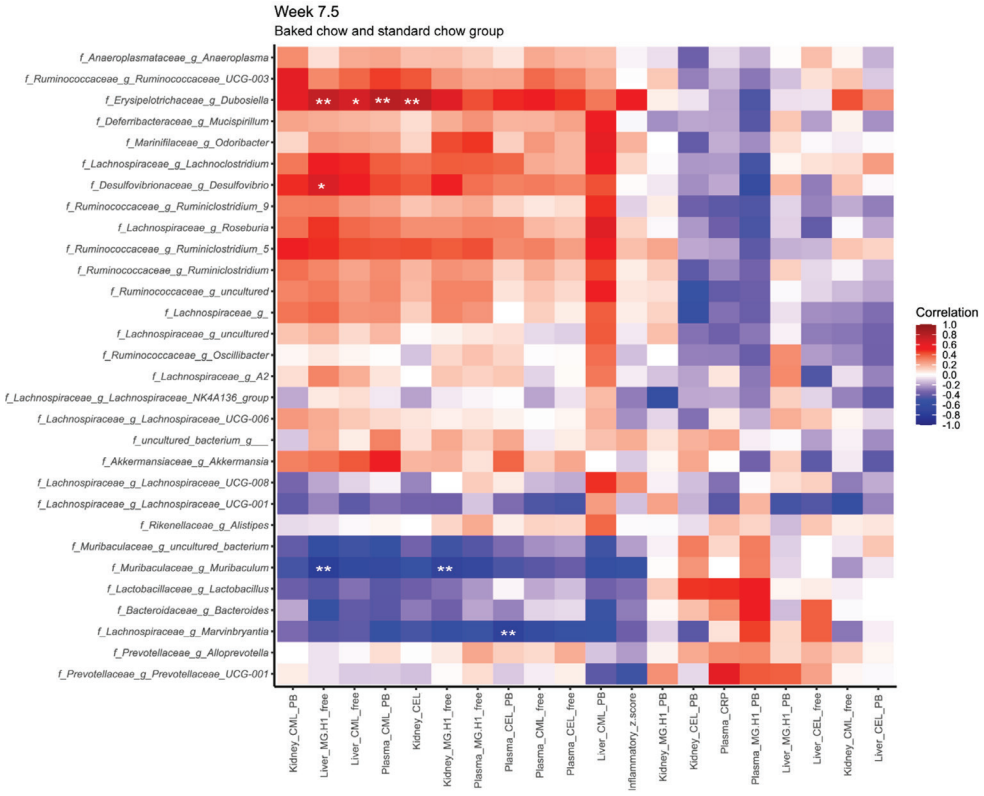
Supplementary Figure 2.4A Bacterial composition plots with the relative abundance of the top 11 taxa at phylum level of each sampling week; taxa are labelled as "other" refer to taxa not present in this top 11. The x-axis represents the mice sampled per diet group (baked chow diet; standard chow diet; switch diet).



Supplementary Figure 2.4B Bacterial composition plots with the relative abundance of the top 11 taxa at genus level of each sampling week; taxa are labelled as "other" refer to taxa not present in this top 11. The x-axis represents the mice sampled per diet group (baked chow diet; standard chow diet; switch diet).



Supplementary Figure 2.5 LEfSe results of the significant taxa found by comparing the mice with the switch diet (red) to the standard chow diet (green), sampled in week 5, 7.5 and 10. Nomenclature was based on the highest achievable taxonomic resolution level. The alpha value was set to 0.05 and the log10 LDA score threshold to 2.0.



Supplementary Figure 2.6 Heatmap of Spearman's rank correlation coefficient of relative abundance microbial composition data of week 7.5 with relevant clinical parameters measured at week 10, of mice in the baked chow and standard chow group. Bacterial taxa with a relative abundance >1% in one of the samples were included. Statistically significant correlations after adjustment for multiple testing are marked with * ($p < 0.1$) or ** ($p < 0.05$). pb: protein-bound.

dietary AGEs in FRIED EGG ON TOAST

CML: 0.9; CEL 1.1; MG-H1: 8.7 mg/100g

Chapter 3

Habitual intake of dietary advanced glycation endproducts is not associated with arterial stiffness of the aorta and carotid artery in adults: The Maastricht Study

Armand M.A. Linkens, Simone J.M.P. Eussen, Alfons J.H.M. Houben, Abraham A. Kroon, Miranda T. Schram, Koen D. Reesink, Pieter C. Dagnelie, Ronald M.A. Henry, Marleen van Greevenbroek, Anke Wesselius, Coen D.A. Stehouwer, Casper G. Schalkwijk

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Abstract

Background: Advanced glycation endproducts (AGEs), a heterogeneous group of bioactive compounds, are thought to contribute to arterial stiffness, which in turn is a causal factor in the pathogenesis of stroke, myocardial infarction, and heart failure. Whether AGEs derived from food also contribute to arterial stiffness is not clear.

Objectives: We investigated whether higher intake of dietary AGEs is associated with arterial stiffness.

Methods: In this cross-sectional observational study in 2255 participants of The Maastricht Study (mean age 60 ± 8 years, 51% male, mean BMI 26.9 ± 4.4 kg/m^2, *n* = 1326 normal glucose metabolism, *n* = 341 prediabetes, and *n* = 585 T2DM), we estimated intake of dietary AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) by a validated food frequency questionnaire coupled to our UPLC-MS/MS dietary AGE database. Arterial stiffness was determined using carotid-femoral pulse wave velocity (cfPWV), carotid distensibility coefficient (carotid DC), and Young's elastic modulus (carotid YEM). We performed multiple linear regression analyses adjusting for potential confounders (demographical, hemodynamic, cardiovascular, and dietary factors).

Results: In the fully adjusted models we observed no statistically significant associations between intake of dietary AGEs CML, CEL, and MG-H1 and arterial stiffness expressed as cfPWV, carotid DC, and carotid YEM.

Conclusions: In adults aged 40-75, habitual intake of dietary AGEs CML, CEL, and MG-H1 is not associated with arterial stiffness measured as cfPWV, carotid DC, or carotid YEM.

Introduction

Advanced glycation endproducts (AGEs) are a heterogeneous group of bioactive compounds formed through the non-enzymatic reaction between reducing sugars and amino acids within proteins and other macromolecules[1]. Plasma AGEs, mainly derived from endogenous formation, are associated with arterial stiffness measured as carotid-femoral (i.e., aortic) pulse wave velocity (cfPWV) and carotid distensibility[2-7]. Arterial stiffness, in turn, is a causal factor in the pathogenesis of stroke, myocardial infarction, and heart failure[8-12]. Endogenously-formed AGEs may lead to arterial stiffness by crosslinking collagen within the arterial wall[13] and activating the receptor for AGEs (RAGE) which subsequently leads to low-grade inflammation[14].

In addition to the endogenous formation, AGEs are abundantly present in sugar and protein rich food items exposed to dry heat[15]. In 450 individuals with an elevated risk for type 2 diabetes mellitus (T2DM) and cardiovascular disease, a higher habitual intake of these dietary AGEs was associated with higher concentrations of AGEs in plasma[16]. Dietary AGEs may have biological effects, as a diet low in AGEs may decrease biomarkers of endothelial dysfunction, low-grade inflammation, and insulin resistance[17]. These effects contribute to arterial stiffening[18, 19]. Despite this, dietary AGEs were not associated with cfPWV in a cross-sectional study and an RCT[20, 21]. However, in these studies dietary AGEs were estimated using a dietary AGE database based on immunoassay methods and comprised only one dietary AGE[22]. Furthermore, sample sizes in both studies were small and only aortic stiffness was assessed.

In view of these considerations, we tested the hypothesis that habitual intake of the specific and well-characterized dietary AGEs CML, CEL, and MG-H1 is associated with arterial stiffness measured both at the aorta and at the carotid artery in a population-based cohort.

Methods

Study design and population

We used data from The Maastricht Study, an observational prospective population-based cohort study. The rationale and methodology have been described previously[23]. In brief, the study focuses on the etiology, pathophysiology, complications, and comorbidities of T2DM and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Participants were recruited through mass media campaigns and from the municipal registries and the

regional Diabetes Patient Registry via mailings. Recruitment was stratified according to known T2DM status for reasons of efficiency. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Netherlands Health Council under the Dutch "Law for Population Studies" (Permit 131088-105234-PG). All participants gave written informed consent. The examinations of each participant were performed within a time window of 3 months. The present report includes cross-sectional data from the first 3451 participants who completed the baseline survey between November 2010 and September 2013. Selection of participants is shown in Figure 3.1.

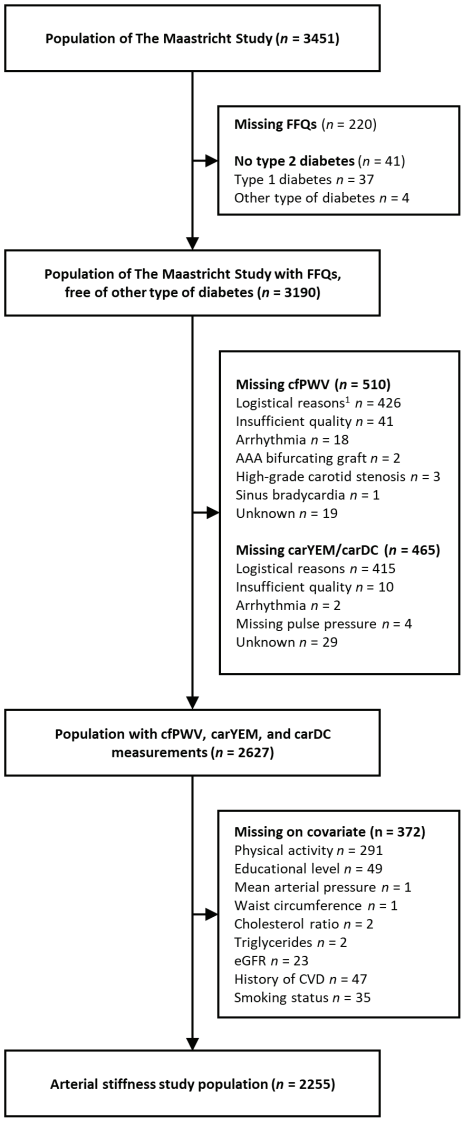

Figure 3.1 Selection of participants from The Maastricht Study cohort. Missings are not mutually exclusive. Abbreviations: AAA, abdominal aorta aneurysm; carDC, carotid Distension Coefficient; carYEM, carotid Young's Elastic Modulus; cfPWV; carotid-to-femoral Pulse Wave Velocity; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate

[1] Logistical reasons: no equipment available, no trained researcher available, technical failure.

Food intake and dietary advanced glycation endproducts

We assessed dietary intake by a validated 253-item FFQ[24]. This FFQ contains 101 questions on consumption with a reference period of one year. The FFQ collected information on the intake of major food groups. All participants filled out the FFQ after their first visit to the study center. Those with implausible energy intake (men: < 800 kcal or > 4200 kcal per day, women: < 500 or > 3500 kcal per day) were excluded from analyses.

Dietary AGE intake was determined by coupling consumption of food items within the FFQ to our dietary AGE database[25]. In this database, three major AGEs, N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) were quantified in protein fractions using highly specific ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). In total, over 200 commonly consumed Western food products are included within this database. For each participant, AGE intake was estimated as described previously[16]. Some of the food products within the FFQ were not analyzed for AGEs. AGE content of these specific products were estimated by matching these products to other comparable products. Only upon return of the FFQ, participants were informed about their glucose metabolism status. As a result, a possible change in dietary habits due to this information will not be reflected within the FFQ.

Measurements of central arterial stiffness

All measurements were carried out by trained vascular technicians unaware of the participants' clinical or diabetes mellitus status. Measurements took place in a quiet, temperature-controlled room (21-23°C) and were performed in supine position, after 10 minutes of rest. Participants were asked to refrain from smoking and drinking coffee, tea or alcoholic beverages 3 hours before measurements. Participants were allowed to have a light meal (breakfast and lunch). Talking or sleeping was not allowed during the examination. A 3-lead ECG was recorded continuously during the measurements to facilitate automatic signal processing. During arterial stiffness measurement, repeated brachial systolic, diastolic, and mean arterial pressures were obtained at 5-minute intervals, using an oscillometric device (Accutorr Plus, Datascope, Inc, Montvale, NJ). The time-averages of systolic, diastolic, and mean arterial pressure were used in the analysis.

Carotid-to-femoral Pulse Wave Velocity

We measured carotid-to-femoral pulse wave velocity (cfPWV) according to recent guidelines[26] with the use of applanation tonometry (SphygmoCor, Atcor Medical, Sydney, Australia). Pressure waveforms were determined at the right common carotid and right common femoral arteries. The difference in the time of pulse arrival from the R-wave of the ECG between the 2 sites (transit time) was determined with the intersecting tangents algorithm. The pulse wave travel distance was calculated as 80% of the direct straight distance (measured with an infantometer) between the 2 arterial sites. cfPWV was defined as travelled distance/transit time. We used the median of 3 consecutive cfPWV recordings in the analyses.

We assessed the reproducibility of cfPWV measurements in 12 individuals (6 men, mean age 60.8 ± 6.8 yr, 6 individuals with T2DM) who were examined by 2 observers on 2 occasions spaced 1 week apart. The intra- and interobserver intraclass correlation coefficients (ICC) were 0.87 and 0.69.

Indices of carotid stiffness

For indices of carotid stiffness, we measured at the left common carotid artery (10 mm proximal to the carotid bulb), with the use of an ultrasound scanner equipped with a 7.5-MHz linear probe (MyLab 70, Esaote Europe B.V., Maastricht, the Netherlands). This set-up enables the measurement of diameter, distension, and intima-media thickness (IMT) as described previously[27, 28]. Briefly, during the ultrasound measurements, a B-mode image based on 19 M-lines was displayed on screen. An online echo-tracking algorithm showed real-time anterior and posterior wall displacements. The multiple M-line recordings were composed of 19 simultaneous recordings at a frame rate of 498 Hz. The distance between the M-line recording positions was 0.96 mm; thus, a total segment of 18.24 mm of each artery was covered by the scan plane. For offline processing, the radiofrequency signal was acquired by a dedicated computer-based system (ART.LAB, Esaote Europe B.V. Maastricht, the Netherlands) with a sampling frequency of 50 MHz. Data processing was performed in MatLab (version 7.5; Mathworks, Natick, Massachusetts, USA). Distension waveforms were obtained from the radiofrequency data by wall tracking, as described in[27]. We defined carotid IMT as the distance of the posterior wall from the leading edge interface between lumen and intima to the leading edge interface between media and adventitia[28]. We used the median diameter, median distension and median IMT of three recordings in the analyses.

Data analysis was done by quantifying the local arterial elastic properties through the

calculation of the following indices[29]:

1. Distensibility coefficient (carDC) = $(2\Delta D \times D + \Delta D^2)/(PP \times D^2)$ $(10^{-3}/kPa)$
2. Young's elastic modulus (carYEM) = D/(IMT × distensibility coefficient) $(10^3$ kPa)

where D is the arterial diameter; ΔD is the distension; IMT the intima–media thickness; and PP the pulse pressure. Local carotid PP was estimated according to the calibration method described by Kelly and Fitchett[30], with the use of carotid tonometry waveforms as adapted by van Bortel et al.[31]. This method assumes a constant difference between MAP and diastolic pressure along the arterial tree. PP can then be calculated at a carotid artery (PPcar) from the uncalibrated carotid pressure waveform using the formula: $PPcar = PPcar_{uncalibrated} \times (Kbrach/Kcar_{uncalibrated})$, in which K is defined as (MAP − diastolic pressure). For the carotid artery, diastolic pressure and MAP are calculated as the minimum and the area under the tonometry waveform divided by time, respectively. The carDC reflects the inverse of arterial stiffness at operating pressure. The carYEM reflects the stiffness of the arterial wall material at operating pressure. Note that increased values of cfPWV or of carYEM, or decreased values of carDC, indicate increased central arterial stiffness.

Glucose Metabolism Status

To determine glucose metabolism, all participants (except those who used insulin and/or had fasting glucose above 11.0 mmol/L) underwent a standardized 7-point oral glucose tolerance test after an overnight fast. Blood samples were taken at baseline and at 15, 30, 45, 60, 90, and 120 minutes after ingestion of a 75 g glucose drink. For safety reasons, participants with a fasting glucose concentration above 11.0 mmol/L, as determined by a finger prick, did not undergo the oral glucose tolerance test. For these individuals, fasting glucose concentration and information about diabetes mellitus medication use were used to determine glucose metabolism status. Glucose metabolism status was defined according to the WHO 2006 criteria into normal glucose tolerance, impaired fasting glucose, impaired glucose tolerance, and T2DM[32]. For this study, we defined having impaired fasting glucose and/or impaired glucose tolerance as prediabetes.

Covariates

Smoking status, history of CVD and physical activity were assessed by a questionnaire. Smoking status was categorized into never, former and current smoker. Waist

circumference, total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), triglycerides, fasting plasma glucose, and glycosylated hemoglobin (HbA1c) were determined as described elsewhere[23]. Estimated glomerular filtration rate (eGFR) was computed with the Chronic Kidney Disease Epidemiology Collaboration formula, using serum creatinine and cystatin C[33]. Information on the use of lipid-modifying and/or antihypertensive medication, that is generic names, doses and frequencies, were collected during an in person medication interview.

Statistical Methods

Analyses were conducted using SPSS version 25 for Windows (IBM Corporation, Armonk, NY, USA). To provide an overview on how participant characteristics differ by dietary AGE intake, baseline characteristics are shown for the total study population and stratified by a dietary AGE score that represents an individuals' overall AGE intake. Because we measured three AGEs in food items which differ in abundance, we first calculated z-scores for all individual dietary AGEs, which were then averaged into a single dietary AGE score. Participants that were excluded from the analyses due to missing covariates were compared with the included participants by means of an ANOVA or chi-squared test, as appropriate.

We performed multiple linear regression to investigate the association between dietary AGEs, expressed standardized on a continuous scale, and indices of arterial stiffness (unstandardized). Three regression models were fitted. In model 1, we adjusted for age (years), sex (male or female), glucose metabolism status (NGM, prediabetes, or T2DM, due to oversampling of individuals with T2DM in The Maastricht Study), and mean heart rate (beats/min) and mean arterial pressure (mmHg) obtained during vascular measurements as these are important determinants of arterial stiffness. In model 2, we additionally adjusted for cardiovascular risk factors as these may be associated with arterial stiffness: waist circumference (cm), total/high-density lipoprotein cholesterol ratio, triglycerides (mmol/L), use of lipid-lowering medication (%-yes), use of anti-hypertensive medication (%-yes), prior CVD (%-yes), kidney function (eGFR), smoking status (former, current, never), and alcohol intake (g/day). Finally, in model 3 we additionally adjusted for lifestyle factors that may be associated with dietary intake: total energy intake (kcal/day), the Dutch Healthy Diet index (DHD-index), educational level (low, middle, or high), and physical activity (hours/week). The DHD-index is a measure of diet quality as it assesses adherence to the Dutch dietary guidelines[34]. A higher index has been associated with more nutrient-dense diets and lower risk of mortality[35, 36]. Crude (model 1) and fully adjusted models (model 3) are presented in the main paper, intermediate models (model 2) in Supplementary tables.

We also performed multiple linear regression analysis with dietary AGEs expressed categorically. For these analyses, dietary AGEs where divided into quartiles, except for stratified analyses, where dietary AGEs where divided into tertiles for reasons of statistical power. To test for linear trend across categories, an ordinal variable with the median value of dietary AGE intake for each quartile/tertile was entered in the regression models. These data are presented in the Supplementary tables.

In addition, we performed interaction analyses for age, sex, glucose metabolism status, and kidney function by adding interaction terms in our model.

We performed several sensitivity analyses. To test the robustness of our results we explored associations between dietary AGEs and arterial stiffness while excluding participants with history of a cardiovascular event, T2DM, or use of antihypertensive medication, as these participants might have altered their diet based on dietary advice from a health care professional. Next, we explored possible confounding of antihypertensive medication after further specification into RAAS inhibitors and other type of antihypertensive medication. We also used ambulatory 24-hour blood pressure measurements (heart rate and mean arterial pressure) instead of blood pressure measurements obtained during the vascular measurements. Additionally, we substituted physical activity data attained from the CHAMPS questionnaire[37] for accelerometer data (ActivPAL). Finally, to assess whether the observed associations were dependent on the food source of AGEs, we performed separate analyses of AGEs from the largest contributing food sources. Beta coefficients are shown with their 95% confidence intervals. *P* values of < 0.05 were considered statistically significant.

Results

Population characteristics

Characteristics of the population with available data on dietary AGEs, arterial stiffness, and covariates are shown in Table 3.1. At a mean intake of 2187 ± 600 kcal, mean daily intakes of the dietary AGEs CML, CEL, and MG-H1 were 3.3 ± 1.1 mg, 3.0 ± 1.2 mg, and 24.4 ± 8.9 mg, respectively. Energy intake was higher and age was slightly lower in the higher dietary AGE quartiles, and these participants consumed more energy as fat and less as protein. In addition, the proportion of men, physical activity, waist circumference, systolic and diastolic blood pressure, and carotid DC were higher in the higher dietary AGE quartiles. The main food groups contributing to AGE intake were cereals, meat, confectionaries, dairy products, and nuts (Supplementary Figure 3.1). Individuals with missing data on vascular measurements or covariates were more likely to have T2DM and to smoke, and individuals with missing data on

covariates had slightly higher cfPWV as compared to individuals included in the study population (Supplementary Table 3.1).

Table 3.1 Baseline characteristics of 2255 adults of The Maastricht Study.

Characteristics	Total population (*n* = 2255)	Dietary AGE quartiles (z-score of all dietary AGEs)[1]			
		Q1 (*n* = 564)	Q2 (*n* = 563)	Q3 (*n* = 565)	Q4 (*n* = 563)
Age (years)	59.9 ± 8.0	60.7 ± 7.7	60.1 ± 8.3	59.7 ± 7.9	59.2 ± 8.0
Sex (n male, %)	1145 (51%)	197 (35%)	267 (47%)	302 (53%)	379 (67%)
Education level (n, %)					
Low	712 (32%)	201 (36%)	156 (27%)	196 (35%)	159 (28%)
Medium	657 (29%)	155 (28%)	171 (30%)	163 (29%)	168 (30%)
High	886 (39%)	208 (37%)	236 (42%)	206 (37%)	236 (42%)
Glucose metabolism status (n, %)					
Normal glucose metabolism	1327 (59%)	317 (56%)	337 (60%)	339 (60%)	334 (59%)
Prediabetes	342 (15%)	86 (15%)	76 (14%)	88 (16%)	92 (16%)
Type 2 diabetes mellitus	586 (26%)	161 (29%)	150 (27%)	138 (24%)	137 (24%)
Smoking (n, %)					
Never	788 (35%)	185 (33%)	208 (37%)	195 (35%)	200 (36%)
Former	1197 (53%)	301 (53%)	288 (51%)	302 (53%)	306 (54%)
Current	270 (12%)	78 (14%)	67 (12%)	68 (12%)	57 (10%)
Physical activity (h/week)	14.3 ± 8.1	14.2 ± 8.1	14.5 ± 8.0	13.4 ± 7.2	15.0 ± 8.8
Waist circumference (cm)	95.4 ± 13.4	94.3 ± 14.2	94.5 ± 13.1	96.1 ± 12.9	96.7 ± 13.3
24-hour Systolic blood pressure (mmHg)	118.8 ± 11.9	117.5 ± 11.7	118.2 ± 12.1	119.6 ± 11.6	120.0 ± 11.7
24-hour Diastolic blood pressure (mmHg)	72.5 ± 7.4	72.5 ± 7.4	72.6 ± 7.1	74.3 ± 7.3	74.5 ± 7.0
Anti-hypertensive medication (n yes, %)	855 (38%)	232 (41%)	213 (38%)	212 (38%)	198 (35%)
Total-to-HDL cholesterol ratio	3.7 ± 1.3	3.6 ± 1.2	3.6 ± 1.2	3.8 ± 1.1	3.8 ± 1.3
Triglycerides (mmol/L)	1.2 (0.9-1.7)	1.2 (0.9-1.7)	1.2 (0.9-1.7)	1.2 (0.9-1.7)	1.2 (0.9-1.7)
Lipid-modifying medication (n yes, %)	796 (35%)	204 (36%)	206 (37%)	192 (34%)	194 (35%)
eGFR (mL/(min·1.73m^2))	88.2 ± 14.4	87.5 ± 14.3	88.5 ± 14.3	87.8 ± 14.3	89.0 ± 14.8
History of cardiovascular disease (n yes, %)	359 (16%)	88 (16%)	92 (16%)	93 (17%)	86 (15%)
Energy intake (kcal/day)	2188 ± 600	1623 ± 372	1993 ± 337	2304 ± 397	2826 ± 508
Carbohydrate (% of energy)	42.8 ± 6.2	43.0 ± 7.0	43.1 ± 6.4	42.7 ± 5.8	42.8 ± 5.5
Fat (% of energy)	34.3 ± 6.0	32.6 ± 6.8	33.8 ± 5.8	35.1 ± 5.6	35.8 ± 5.1
Protein (% of energy)	15.9 ± 2.5	16.5 ± 2.9	16.1 ± 2.6	15.8 ± 2.2	15.3 ± 2.2
Fiber (% of energy)	2.5 ± 0.6	2.7 ± 0.7	2.5 ± 0.5	2.5 ± 0.5	2.4 ± 0.5
Alcohol (g/day)	8.5 (1.5-18.8)	7.2 (0.6-17.5)	8.4 (1.5-17.6)	8.7 (2.0-17.4)	9.4 (2.5-21.0)
Dutch Healthy Diet Index	83.6 ± 14.6	86.3 ± 14.7	84.8 ± 13.9	82.6 ± 14.7	80.7 ± 14.4
Dietary CML (mg/day)	3.3 ± 1.1	2.1 ± 0.5	2.9 ± 0.4	3.5 ± 0.4	4.7 ± 1.0
Dietary CEL (mg/day)	3.0 ± 1.2	1.8 ± 0.4	2.5 ± 0.3	3.1 ± 0.4	4.5 ± 1.4
Dietary MG-H1 (mg/day)	24.4 ± 8.9	15.6 ± 3.1	21.0 ± 2.4	25.7 ± 3.0	35.3 ± 9.3
Carotid-femoral Pulse Wave Velocity (m/s)	9.0 ± 2.1	9.1 ± 2.2	9.0 ± 2.2	8.8 ± 1.9	8.9 ± 2.1
Carotid Distensibility Coefficient (mm^2/kPa)	14.3 ± 5.1	13.8 ± 5.0	14.5 ± 5.2	14.5 ± 5.2	15.6 ± 5.0
Carotid Young's Elastic Modulus (10^3/kPa)	0.7 ± 0.3	0.8 ± 0.3	0.7 ± 0.4	0.7 ± 0.4	0.7 ± 0.4

Values are presented as means ± SD, medians (interquartile range), or frequencies (*n*, %), as appropriate. Abbreviations: CEL, N^{ε}-(1-carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; eGFR, estimated glomerular filtration rate; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Data are shown without stratification, and according to quartiles of a z-score representing overall dietary AGE intake.

Intake of dietary AGEs and arterial stiffness

In continuous analyses, dietary AGEs CML, CEL, and MG-H1 were not associated with cfPWV, carDC, or carYEM in any of the models (Table 3.2). When expressing dietary AGEs as quartiles, overall associations were similar, except for the associations between MG-H1 and cfPWV, and between CEL and carYEM (Supplementary Table 3.2). In models adjusted for participant characteristics and cardiovascular factors, MG-H1 intake > 28.6 mg/day (Q4) was associated with a lower cfPWV compared to MG-H1 intake < 18.5 mg/day (Q1) (β -0.20 m/s; 95% CI: -0.40,0.00, *p* for linear trend < 0.04), but this association lost statistical significance after adjustment for lifestyle factors in the final model (β-0.04 m/s; 95% CI:-0.32,0.23, *p* for linear trend = 0.77). Contrarily, carYEM was slightly lower among those in Q3 and Q4 of CEL intake (2.8-3.5 mg/day and > 3.5 mg/day) compared to those in Q1 (< 2.2 mg/day) in fully adjusted models (for Q3: β-0.05 10^3/kPa; 95% CI:-0.09,-0.01 and for Q4: β-0.05 10^3/kPa; 95% CI:-0.10,-0.00, *p* for linear trend = 0.05) (Supplementary Table 3.2).

Of note, in the fully adjusted models greater total energy intake was independently associated with lower cfPWV, but not with carDC or carYEM (data not shown).

Table 3.2 Associations between dietary AGEs and arterial stiffness in 2255 adults of The Maastricht Study

Dietary AGE (SD/day)	**cfPWV** (m/s)	**Carotid DC** (mm^2/kPa)	**Carotid YEM** (10^3/kPa)
CML			
Semi-adjusted β (95% CI)[1]	-0.05 (-0.12,0.02)	-0.14 (-0.31,0.04)	0.01 (-0.01,0.02)
Fully-adjusted β (95% CI)[2]	0.04 (-0.07,0.15)	-0.16 (-0.43,0.11)	0.01 (-0.01,0.03)
CEL			
Semi-adjusted β (95% CI)[1]	-0.01 (-0.08,0.06)	-0.06 (-0.23,0.11)	0.00 (-0.01,0.01)
Fully-adjusted β (95% CI)[2]	0.05 (-0.04,0.14)	-0.02 (-0.23,0.20)	-0.01 (-0.02,0.01)
MG-H1			
Semi-adjusted β (95% CI)[1]	-0.06 (-0.13,0.01)	-0.13 (-0.30,0.05)	0.01 (-0.01,0.02)
Fully-adjusted β (95% CI)[2]	0.00 (-0.09,0.10)	-0.11 (-0.35,0.13)	0.00 (-0.01,0.02)

Abbreviations: AGE, advanced glycation endproduct; Carotid DC, carotid distension coefficient; CarYEM, carotid Young's Elastic Modulus; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Regression coefficients (β) and 95% CI represent the change in arterial stiffness measurement per 1-SD change in dietary AGE intake while adjusted for age, sex, glucose metabolism status, heart rate, and mean arterial pressure obtained during vascular measurements.

[2] Additionally adjusted for waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, kidney function, energy intake, educational level, physical activity, and the Dutch Healthy Diet index.

Intake of dietary AGEs and arterial stiffness in subgroups of glucose metabolism

Tests for interaction revealed that glucose metabolism status significantly modified the associations between dietary AGEs and cfPWV (all *P* for interactions < 0.05), but not with carYEM and carDC (all *p* for interactions > 0.05). However, also when stratified for glucose metabolism status dietary AGEs were not associated with cfPWV in the fully adjusted models (Supplementary Table 3.3 and 3.4). Sex, age, and kidney function showed no significant interactions for the association between dietary AGEs and measurements of arterial stiffness (all *p* for interactions > 0.05, data not shown).

Additional analyses

When excluding participants with T2DM, use of antihypertensive medication, or a known history of CVD, positive associations between dietary AGEs and cfPWV observed earlier in participants with normal glucose metabolism were attenuated (Supplementary Table 3.5). Likewise, while greater intake of CML and MG-H1 were associated with lower carotid DC and greater carotid YEM in intermediate models, statistical significance was again lost in the fully adjusted models (Supplementary Table 3.5). Greater intake of CEL was borderline significantly associated with lower carotid YEM in the fully adjusted model (Supplementary Table 3.5). Categorical analyses were largely in agreement, except for greater carotid DC in tertile 2 of CML intake compared to tertile 1 in the fully adjusted model (Supplementary Table 3.5).

Next, we substituted physical activity obtained from the CHAMPS questionnaire for accelerometer data, which was available in less participants. Although physical activity did not act as a confounder in these analyses, participants in the highest quartile of CEL intake no longer showed lower carotid YEM compared to those in the lowest quartile (Supplementary Table 3.6). Associations between dietary AGEs and carotid DC were unchanged (data not shown). Participants with T2DM in the highest tertile of MG-H1 intake showed lower cfPWV when compared to those in the lowest tertile, even after adjusting for lifestyle factors (Supplementary Table 3.7). All other sensitivity analyses did not materially change the results (data not shown).

Lastly, we investigated associations between dietary AGEs from food groups and arterial stiffness. In line with overall intake of dietary AGEs, dietary AGEs from these subgroups were not associated with cfPWV or carotid YEM (Supplementary Table 3.8 and 3.9). In contrast, greater intake of CML and CEL from cereals were associated with lower carotid DC in the fully adjusted models: β-0.27 mm^2/kPa; 95% CI:-0.47,-0.07 for CML, and β-0.23 mm^2/kPa; 95% CI:-0.42,-0.04 for CEL (Supplementary Table 3.10). Dietary AGEs from all other food sources were not associated with carotid DC.

Discussion

This is the first study on intake of dietary AGEs CML, CEL, and MG-H1 and arterial stiffness in a population-based setting. Results showed no association of dietary AGEs with arterial stiffness measured as either cfPWV, carotid DC, or carotid YEM, after full adjustment for potential confounders.

Our findings are largely in agreement with two previous studies in smaller groups. In a cross-sectional study, Di Pino et al. found a positive association between dietary CML and augmentation index, but not cfPWV, in a group of 85 CVD- and complication-free T2DM patients in adjusted analyses[20]. In line with this, in an RCT, no difference in cfPWV was observed in 62 participants with prediabetes who followed a 24-week dietary regimen either low or standard in dietary AGEs[21]. However, some methodological drawbacks may explain these null findings. Especially for the observational study, the small sample size did not enable adequate correction for possible confounders, although this information was available. Also, dietary AGEs in both studies were measured by ELISA[22], a method that has been shown to quantify low AGE food products as being high in AGEs, and vice versa, when compared to the gold standard UPLC-MS/MS technique[38]. For the intervention trial, it is possible that the difference in dietary AGEs between groups therefore was too small, especially because one of the groups was advised to continue their normal dietary habits. Furthermore, only dietary CML was addressed in both studies. We circumvented these methodological limitations by investigating not only dietary CML, but also CEL and MG-H1, in relation to arterial stiffness, which were measured in food items by UPLC-MS/MS[25]. These dietary AGEs differ in abundance and structure and may therefore also have different biological effects[39]. In addition, the sample size in the current study was much larger and included substantial numbers of individuals with normal glucose metabolism, prediabetes, and T2DM.

While cfPWV is considered the gold standard method to determine arterial stiffness, it is important to address both aortic and carotid stiffness, as they are affected differently by cardiovascular risk factors[40], but both predict CVD[8, 11]. We observed a small but statistically significant inverse association between quartiles of CEL intake and carotid YEM in the fully adjusted models. However, this observation was not consistent in our sensitivity analyses. There were no associations between the other dietary AGEs and carotid YEM or carotid DC.

We recently showed, in a smaller selection of the current population, that protein-bound pentosidine in plasma and skin autofluoresence (SAF) are associated with higher cfPWV[4]. Both protein-bound pentosidine in plasma and SAF are regarded

as reflections of collagen cross-linking in the vascular wall, which leads to arterial stiffness by directly altering structural properties of a vessel. In humans, it is unknown whether dietary AGEs accumulate in tissues such as the vascular wall. In mice, dietary AGEs accumulate in several organs as shown after chronic exposure to labeled dietary AGEs[41]. However, the accumulation of dietary AGEs in plasma and tissues is in its free form and not the protein bound form, which is in line with the hypothesis that dietary AGEs, consumed as whole proteins, undergo digestion and subsequently enter the circulation as free- or peptide-bound AGEs[16]. It is unlikely that the free form of AGEs is involved in collagen cross-linking since this process occurs during endogenous formation of AGEs in collagen. Aside from collagen-crosslinking, AGEs may be involved in arterial stiffness by activation of the receptor for AGEs (RAGE)[42, 43]. In line with this, mice fed a CML-enriched diet for 9 months developed arterial stiffness, an observation that was not seen in RAGE-knockout mice fed the same diet[44]. However, whether AGE-RAGE interactions play a role in vascular effects of dietary AGEs in humans is a matter of debate. Only protein-bound AGEs have been reported to have affinity for RAGE, while free or peptide-bound AGEs do not[45]. This, combined with the findings of the present study and those mentioned previously, suggests that dietary AGEs do not share the role of endogenously-formed AGEs in arterial stiffness. However, dietary AGEs may have implications beyond arterial stiffness. Consequences of a diet high in AGEs include increased plasma markers of inflammation and endothelial dysfunction. An interesting target for further research is the microcirculation, blood vessels with a diameter less than 150 μm[46]. Because 98% of all endothelial surface resides within the microcirculation, dietary AGE-induced inflammation and endothelial dysfunction may have consequences for microvascular function.

The intake of AGEs in the current cohort is comparable to that in other cohorts were dietary AGEs were assessed using UPLC-MS/MS. In individuals at high risk for cardiovascular disease and T2DM of the CODAM study, we estimated mean daily intake of CML, CEL, and MG-H1 at 3.1 ± 1.0 mg/day, 2.3 ± 0.8 mg/day, and 21.7 ± 6.7 mg/day[16]. The contribution of different food groups to overall AGE intake were largely comparable to that measured in a large sub cohort of the EPIC study[47]. Additionally, analyses stratified for food groups were largely in agreement with analyses using overall dietary AGE intake, suggesting that our observations were not driven by any of the individual food groups. Although we found that greater intake of CML and CEL from cereals was associated with less carotid DC, indicating greater carotid stiffness, this finding should be interpreted with caution. Cereals represent a wide group of grain products that may differ in cardiovascular properties[48]. Due to the absence of an overall pattern in these additional analyses and the large quantity of statistical tests this may reflect a false positive finding.

Interestingly, adjusting for total energy intake greatly attenuated the associations between dietary AGEs and cfPWV, and greater total energy intake was associated with lower cfPWV in the fully adjusted models, independent of physical activity. This is a surprising, as it has been shown that lower total energy intake is associated with lower arterial stiffness[49], while the opposite has not yet been described. Although we cannot explain the underlying mechanism, this underlines the importance of adjusting for total energy intake in nutritional epidemiology and researchers investigating the role of dietary AGEs in arterial stiffening should consider this association when performing their analyses.

Our study has several strengths. The extensive phenotyping and population-based approach of The Maastricht Study enables adjustment for important confounders. Furthermore, gold standard methods were applied where possible: AGEs in food items were measured with the highly specific UPLC-MS/MS technique, and arterial stiffness using recent guidelines[50]. The Maastricht Study FFQ, although not a gold standard method, has been validated against 24-hour recalls and several biomarkers[24].

Our study also has several limitations. Primarily, the current study is observational and while we adjusted for many potential confounders, we cannot exclude the possibility of residual confounding. In addition, The Maastricht Study mainly includes middle-aged Caucasians. Applying our results to other populations, especially with different dietary habits, should be performed with caution. Additionally, roughly 1/3 of our participants were excluded from the analysis due to missing information on FFQs, arterial stiffness, or covariates. The main reason for this were missing arterial stiffness measurements due to logistical reasons (being mainly no trained personnel or no device available) that occurred by chance. It is therefore unlikely that this has resulted in selection bias to an extent that it has influenced the current results. A drawback of our FFQ and dietary AGE database is that they do not include detailed information about food preparation. Different cooking techniques and heating duration determine to a large extent the quantity of AGEs in food[51]. Despite this, the reliability of dietary AGE assessment was increased by the addition of several high AGE products in the FFQ (i.e. blood sausage, toasted bread, beef stew). Another limitation is that FFQs are prone to recall bias[52]. However, to date there is no reliable biomarker for dietary AGE intake. Furthermore, we previously found an association between dietary AGEs measured with FFQs and free AGEs in plasma in a different cohort, indicating that the combination of a FFQ and our dietary AGE database can be used to assess habitual dietary AGE intake and subsequently rank participants according to their AGE intake.

In conclusion, habitual intake of the dietary AGEs CML, CEL, and MG-H1 is not associated with either cfPWV, carotid DC, or carotid YEM in these cross-sectional analyses. Additional RCTs in which dietary AGEs are accurately assessed by UPLC-MS/MS and shortcomings of an FFQ are circumvented are needed to fully elucidate the role of dietary AGEs in arterial stiffening. Furthermore, the implications of dietary AGEs on other outcomes, such as microvascular function, deserves further investigation.

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Supplementary Methods

24-hour Ambulatory blood pressure measurement

Ambulatory blood pressure was measured with ambulatory 24-hour BP monitoring (WatchBP O3, Microlife AG, Switzerland). Cuffs were applied to the participants' non-dominant arm. Measurements were programmed for every 15 minutes during daytime (08.00–23.00 hours) and every 30 minutes during the night (23.00–08.00 hours), for a total of 24 hours. As quality criteria, mean 24-hour blood pressure measurements were only calculated if more than 14 valid measurements at daytime and more than 7 valid measurements at night were available, based on recommendations of the British Hypertension Society[1]. 24-hour ambulatory heart rate, and 24-hour ambulatory mean arterial pressure (MAP, defined as aDBP + (0.412 x aPP))[2] were calculated based on hourly averages[3].

Accelerometer data

Physical activity was measured using the activPAL3 physical activity monitor (PAL Technologies, Glasgow, UK). The activPAL3 is a small (53×35×7 mm), lightweight (15 g) triaxial accelerometer that records movement in the vertical, anteroposterior and mediolateral axes, and also determines posture (sitting or lying, standing and stepping) based on acceleration information. The device was attached directly to the skin on the front of the right thigh with transparent 3M Tegaderm tape, after the device had been waterproofed using a nitrile sleeve. Participants were asked to wear the accelerometer for 8 consecutive days, without removing it at any time. To avoid inaccurately identifying non-wear time, participants were asked not to replace the device once removed. Data were uploaded using the activPAL software and processed using customized software written in MATLAB R2013b (MathWorks, Natick, MA, USA). Data from the first day were excluded from the analysis because participants performed physical function tests at the research centre after the device was attached. In addition, data from the final wear day providing ≤14 waking hours of data were excluded from the analysis. Participants were included if they provided at least 1 valid day (>14 h of waking data).

References to Supplementary Methods

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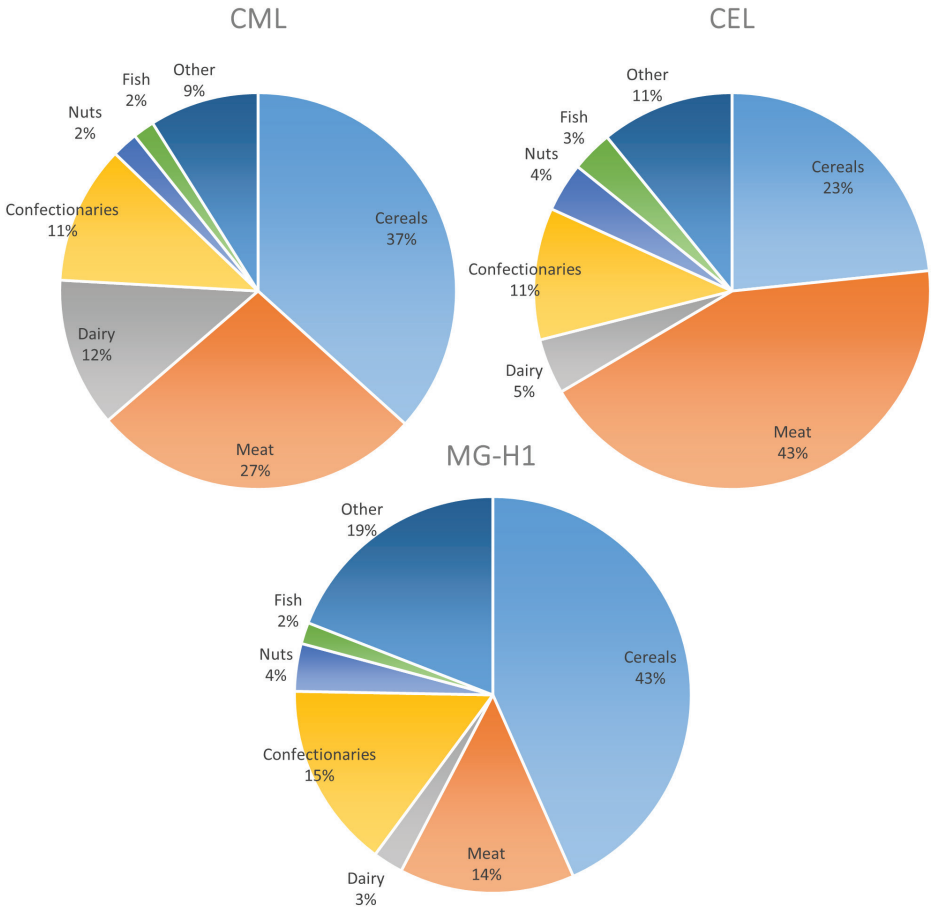
Supplementary Figures



Supplementary Figure 3.1 Contribution of food groups to AGE intake in 2255 adults of The Maastricht Study. Abbreviations: AGE, advanced glycation endproduct; CEL, N^{ε}-(1-carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Supplementary Tables

Supplementary Table 3.1 Characteristics of participants included in the analyses compared to those excluded.

Characteristics	Arterial stiffness population (*n* = 2255)	Excluded due to missing (*n* = 1196)	*P*-value[1]
Age (years)	59.9 ± 8.0	59.5 ± 8.8	0.14
Sex (% male)	1145 (51%)	630 (53%)	0.29
Education level (n, %)			<0.01
Low	712 (32%)	423 (38%)	
Medium	657 (29%)	297 (26%)	
High	886 (39%)	402 (36%)	
Glucose metabolism status (n, %)			<0.01
Normal glucose metabolism	1327 (59%)	597 (50%)	
Prediabetes	342 (15%)	169 (14%)	
Type 2 diabetes mellitus	586 (26%)	389 (33%)	
Other[2]	0 (0%)	41 (3%)	
Smoking (n, %)			<0.01
Never	788 (35%)	384 (34%)	
Former	1197 (15%)	562 (49%)	
Current	270 (26%)	200 (17%)	
Physical activity (h/week)	14.3 ± 8.1	13.8 ± 8.3	0.13
Waist circumference (cm)	95.4 ± 13.4	97.0 ± 14.5	<0.01
24-hour Systolic blood pressure (mmHg)	118.8 ± 11.9	119.6 ± 11.6	0.10
24-hour Diastolic blood pressure (mmHg)	72.5 ± 7.4	73.5 ± 6.7	0.80
Anti-hypertensives (n yes, %)	855 (38%)	537 (45%)	<0.01
Total-to-HDL cholesterol ratio	3.7 ± 1.2	3.6 ± 1.2	0.27
Triacylglycerides (mmol/L)	1.2 (0.9-1.7)	1.2 (0.9-1.8)	0.46
Lipid-modifying medication (n yes, %)	796 (35%)	463 (39%)	<0.05
eGFR (mL(min·1.73m^2))	88.2 ± 14.4	88.0 ± 15.9	0.67
History of cardiovascular disease (n yes, %)	359 (16%)	209 (19%)	0.06
Energy intake (kcal/day)	2188 ± 600	2167 ± 622	0.39
Carbohydrate (% of energy)	42.8 ± 6.2	42.9 ± 6.2	0.94
Fat (% of energy)	34.3 ± 6.0	34.4 ± 6.2	0.78
Protein (% of energy)	15.9 ± 2.5	16.1 ± 2.8	0.07
Fiber (% of energy)	2.5 ± 0.6	2.5 ± 0.6	0.99
Alcohol (g/day)	8.5 (1.5-18.8)	8.0 (1.0-18.4)	0.10
Dutch Healthy Diet Index	83.6 ± 14.6	82.4 ± 15.0	0.03
Dietary CML (mg/day)	3.3 ± 1.1	3.3 ±1.1	0.41
Dietary CEL (mg/day)	3.0 ± 1.2	3.0 ± 1.2	0.85
Dietary MG-H1 (mg/day)	24.4 ± 8.9	24.1 ± 8.3	0.37
Carotid-femoral Pulse Wave Velocity (m/s)	9.0 ± 2.1	9.2 ± 2.4	<0.01
Carotid Distensibility Coefficient (mm^2/kPa)	14.3 ± 5.1	14.3 ± 5.2	0.73
Carotid Young's Elastic Modulus (10^3/kPa)	0.7 ± 0.3	0.8 ± 0.5	0.03

Data are presented as means ± SD, medians (interquartile range), or frequencies (n, %), as appropriate. Abbreviations: CEL, N^{ε}-(1-carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; eGFR, estimated glomerular filtration rate; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Comparisons between groups were performed with ANOVA or X^2, as appropriate.

[2] Other types of diabetes: Type 1 diabetes mellitus, surgery or medicine-induced diabetes mellitus.

Supplementary Table 3.2 Associations between dietary AGEs and arterial stiffness in 2255 adults of The Maastricht Study.

	Continuous	Quartile 1 (ref) *n* = 564	Quartile 2 *n* = 564	Quartile 3 *n* = 564	Quartile 4 *n* = 564	P_{trend}
cfPWV (m/s)						
CML	SD/day	<2.5 mg/day	2.5-3.1 mg/day	3.1-3.9 mg/day	>3.9 mg/day	
Model 1 β (95% CI)[1]	-0.05 (-0.12,0.02)	0	**-0.21 (-0.40,-0.01)**	-0.17 (-0.37,0.03)	-0.19 (-0.38,0.02)	0.14
Model 2 β (95% CI)[2]	-0.06 (-0.13,0.02)	0	**-0.21 (-0.41,-0.01)**	-0.18 (-0.37,0.02)	-0.20 (-0.40,0.01)	0.12
Model 3 β (95% CI)[3]	0.04 (-0.07,0.15)	0	-0.14 (-0.34,0.07)	-0.05 (-0.28,0.18)	0.02 (-0.27,0.31)	0.68
CEL	SD/day	<2.2 mg/day	2.2-2.8 mg/day	2.8-3.5 mg/day	>3.5 mg/day	
Model 1 β (95% CI)[1]	-0.01 (-0.08,0.06)	0	-0.02 (-0.22,0.18)	-0.16 (-0.36,0.04)	-0.12 (-0.32,0.09)	0.17
Model 2 β (95% CI)[2]	-0.02 (-0.09,0.05)	0	-0.01 (-0.21,0.18)	-0.18 (-0.37,0.02)	-0.13 (-0.33,0.07)	0.12
Model 3 β (95% CI)[3]	0.05 (-0.04,0.14)	0	0.05 (-0.16,0.25)	-0.08 (-0.29,0.15)	0.07 (-0.21,0.30)	0.91
MG-H1	SD/day	<18.5 mg/day	18.5-23.1 mg/day	23.1-28.6 mg/day	>28.6 mg/day	
Model 1 β (95% CI)[1]	-0.06 (-0.13,0.01)	0	-0.11 (-0.31,0.08)	-0.18 (-0.38,0.02)	**-0.21 (-0.42,-0.02)**	**0.03**
Model 2 β (95% CI)[2]	-0.06 (-0.13,0.01)	0	-0.10 (-0.30,0.10)	-0.18 (-0.38,0.02)	**-0.20 (-0.40,-0.00)**	**0.04**
Model 3 β (95% CI)[3]	0.00 (-0.09,0.10)	0	-0.05 (-0.26,0.16)	-0.09 (-0.31,0.14)	-0.04 (-0.32,0.23)	0.77
carDC (mm^2/kPa)						
CML	SD/day	<2.5 mg/day	2.5-3.1 mg/day	3.1-3.9 mg/day	>3.9 mg/day	
Model 1 β (95% CI)[1]	-0.14 (-0.31,0.04)	0	0.29 (-0.19,0.77)	0.09 (-0.39,0.57)	-0.22 (-0.71,0.28)	0.23
Model 2 β (95% CI)[2]	-0.12 (-0.30,0.05)	0	0.30 (-0.18,0.78)	0.13 (-0.35,0.61)	-0.16 (-0.65,0.34)	0.32
Model 3 β (95% CI)[3]	-0.16 (-0.43,0.11)	0	0.32 (-0.18,0.83)	0.16 (-0.40,0.71)	-0.11 (-0.81,0.60)	0.57
CEL	SD/day	<2.2 mg/day	2.2-2.8 mg/day	2.8-3.5 mg/day	>3.5 mg/day	
Model 1 β (95% CI)[1]	-0.06 (-0.23,0.11)	0	0.01 (-0.47,0.49)	0.14 (-0.34,0.62)	0.10 (-0.40,0.59)	0.64
Model 2 β (95% CI)[2]	-0.05 (-0.23,0.12)	0	0.00 (-0.47,0.48)	0.19 (-0.29,0.67)	0.13 (-0.36,0.63)	0.49
Model 3 β (95% CI)[3]	-0.02 (-0.23,0.20)	0	0.12 (-0.38,0.61)	0.38 (-0.15,0.91)	0.45 (-0.17,1.08)	0.12
MG-H1	SD/day	<18.5 mg/day	18.5-23.1 mg/day	23.1-28.6 mg/day	>28.6 mg/day	
Model 1 β (95% CI)[1]	-0.13 (-0.30,0.05)	0	0.27 (-0.21,0.74)	-0.11 (-0.59,0.37)	-0.14 (-0.63,0.35)	0.31
Model 2 β (95% CI)[2]	-0.12 (-0.30,0.05)	0	0.24 (-0.24,0.72)	-0.10 (-0.58,0.38)	-0.12 (-0.60,0.37)	0.38
Model 3 β (95% CI)[3]	-0.11 (-0.35,0.13)	0	0.30 (-0.20,0.80)	-0.02 (-0.57,0.54)	0.04 (-0.63,0.71)	0.86
carYEM (10^3/kPa)						
CML	SD/day	<2.5 mg/day	2.5-3.1 mg/day	3.1-3.9 mg/day	>3.9 mg/day	
Model 1 β (95% CI)[1]	0.01 (-0.01,0.02)	0	-0.02 (-0.05,0.02)	-0.00 (-0.04,0.03)	0.02 (-0.02,0.05)	0.28
Model 2 β (95% CI)[2]	0.01 (-0.01,0.02)	0	-0.02 (-0.05,0.02)	-0.01 (-0.04,0.03)	0.01 (-0.02,0.05)	0.37
Model 3 β (95% CI)[3]	0.01 (-0.01,0.03)	0	-0.02 (-0.05,0.02)	-0.01 (-0.05,0.04)	0.01 (-0.04,0.06)	0.54
CEL	SD/day	<2.2 mg/day	2.2-2.8 mg/day	2.8-3.5 mg/day	>3.5 mg/day	
Model 1 β (95% CI)[1]	0.00 (-0.01,0.01)	0	-0.03 (-0.06,0.01)	-0.03 (-0.07,0.01)	-0.02 (-0.06,0.02)	0.36
Model 2 β (95% CI)[2]	-0.00 (-0.01,0.01)	0	-0.03 (-0.06,0.01)	-0.03 (-0.07,0.00)	-0.02 (-0.06,0.01)	0.27
Model 3 β (95% CI)[3]	-0.01 (-0.02,0.01)	0	-0.03 (-0.07,0.00)	-0.05 (-0.09,-0.01)	**-0.05 (-0.10,-0.00)**	**0.05**
MG-H1	SD/day	<18.5 mg/day	18.5-23.1 mg/day	23.1-28.6 mg/day	>28.6 mg/day	
Model 1 β (95% CI)[1]	0.01 (-0.01,0.02)	0	-0.01 (-0.05,0.02)	-0.01 (-0.03,0.04)	-0.01 (-0.03,0.05)	0.73
Model 2 β (95% CI)[2]	0.01 (-0.01,0.02)	0	-0.01 (-0.05,0.03)	0.00 (-0.03,0.04)	0.01 (-0.03,0.05)	0.47
Model 3 β (95% CI)[3]	0.00 (-0.01,0.02)	0	-0.01 (-0.05,0.03)	0.00 (-0.04,0.04)	0.00 (-0.05,0.05)	0.79

Data are shown according to quartiles of AGE intake and AGEs expressed continuously. Abbreviations: AGE, advanced glycation endproduct; CarDC, carotid distension coefficient; CarYEM, carotid Young's Elastic Modulus; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Regression coefficients (β) and 95% CI represent the difference in arterial stiffness (in m/s for cfPWV, mm^2/kPa for carotid DC, and 10^3/kPa for carotid YEM) per 1-SD change in dietary AGE intake or for a dietary AGE quartile compared to the reference quartile while adjusted for age, sex, glucose metabolism status, and heart rate and mean arterial pressure obtained during vascular measurements.

[2] Additionally adjusted for: waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, and kidney function.

[3] Additionally adjusted for: energy intake, educational level, physical activity and the Dutch Healthy Diet index.

Supplementary Table 3.3 Associations between dietary AGEs and cfPWV (m/s) in adults of The Maastricht Study by glucose metabolism status

Dietary AGE (SD/day)	NGM *n* = 1327	Prediabetes *n* = 342	T2DM *n* = 586	$P_{interaction}$ [3]	$P_{interaction}$ [4]
CML					
Semi-adjusted β (95% CI)[1]	0.04 (-0.05,0.13)	**-0.21 (-0.42,-0.02)**	-0.10 (-0.26,0.05)		
Fully-adjusted β (95% CI)[2]	0.09 (-0.05,0.23)	-0.04 (-0.32,0.24)	0.04 (-0.20,0.28)	<0.01	0.01
CEL					
Semi-adjusted β (95% CI)[1]	0.07 (-0.02,0.16)	-0.11 (-0.29,0.07)	-0.06 (-0.21,0.09)		
Fully-adjusted β (95% CI)[2]	0.10 (-0.02,0.21)	0.00 (-0.21,0.22)	0.03 (-0.15,0.21)	<0.01	0.02
MG-H1					
Semi-adjusted β (95% CI)[1]	-0.01 (-0.10,0.08)	-0.11 (-0.28,0.07)	-0.11 (-0.26,0.05)		
Fully-adjusted β (95% CI)[2]	-0.03 (-0.16,0.11)	0.04 (-0.17,0.26)	0.01 (-0.20,0.22)	0.01	0.05

Abbreviations: AGE, advanced glycation endproduct; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; NGM, normal glucose metabolism; T2DM, type 2 diabetes mellitus.

[1] Regression coefficients (β) and 95% CI represent the change in cfPWV (in m/s) per 1-SD change in dietary AGE intake while adjusted for age, sex, and heart rate and mean arterial pressure obtained during vascular measurements

[2] Additionally adjusted for waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, kidney function, energy intake, educational level, physical activity and the Dutch Healthy Diet index.

[3] Interaction for NGM versus prediabetes, only tested in the fully-adjusted model

[4] Interaction for NGM versus T2DM, only tested in the fully-adjusted model

Supplementary Table 3.4 Associations between dietary AGEs as tertiles and cfPWV (m/s) in adults of The Maastricht Study by glucose metabolism status.

	Continuous	Tertile 1(ref)	Tertile 2	Tertile 3	P_{trend}
CML					
Normal glucose metabolism	SD/day	<2.7 mg/day	2.7-3.6 mg/day	>3.6 mg/day	
Sample size	1327	442	443	442	
Model 1 β (95% CI)[1]	0.04 (-0.05,0.13)	0	0.07 (-0.12,0.26)	0.11 (-0.09,0.31)	0.28
Model 2 β (95% CI)[2]	0.05 (-0.04,0.14)	0	0.08 (-0.11,0.27)	0.13 (-0.07,0.33)	0.20
Model 3 β (95% CI)[3]	0.09 (-0.05,0.23)	0	0.12 (-0.09,0.33)	0.20 (-0.07,0.48)	0.16
Prediabetes	SD/day	<2.8 mg/day	2.8-3.7 mg/day	>3.7 mg/day	
Sample size	342	114	114	114	
Model 1 β (95% CI)[1]	**-0.21 (-0.42,-0.02)**	0	-0.34 (-0.85,0.16)	-0.50 (-1.01,0.01)	**0.05**
Model 2 β (95% CI)[2]	**-0.21 (-0.41,-0.02)**	0	-0.33 (-0.84,0.19)	-0.49 (-1.01,0.01)	0.06
Model 3 β (95% CI)[3]	-0.04 (-0.32,0.24)	0	-0.15 (-0.71,0.40)	-0.07 (-0.76,0.62)	0.86
T2DM	SD/day	<2.7 mg/day	2.7-3.6 mg/day,	>3.6 mg/day	
Sample size	586	195	196	195	
Model 1 β (95% CI)[1]	-0.10 (-0.26,0.05)	0	-0.34 (-0.75,0.06)	-0.39 (-0.80,0.03)	0.08
Model 2 β (95% CI)[2]	-0.12 (-0.28,0.03)	0	-0.38 (-0.79,0.02)	**-0.44 (-0.85,-0.02)**	**0.05**
Model 3 β (95% CI)[3]	0.04 (-0.20,0.28)	0	-0.22 (-0.67,0.23)	-0.17 (-0.76,0.42)	0.63
CEL					
Normal glucose metabolism	SD/day	<2.4 mg/day	2.4-3.4 mg/day	>3.4 mg/day	
Sample size	1327	442	443	442	
Model 1 β (95% CI)[1]	0.07 (-0.02,0.16)	0	0.03 (-0.16,0.22)	0.02 (-0.18,0.21)	0.89
Model 2 β (95% CI)[2]	0.07 (-0.02,0.16)	0	0.01 (-0.18,0.20)	0.01 (-0.19,0.20)	0.97
Model 3 β (95% CI)[3]	0.10 (-0.02,0.21)	0	0.00 (-0.20,0.20)	-0.03 (-0.27,0.22)	0.83
Prediabetes	SD/day	<2.4 mg/day	2.4-3.3 mg/day	>3.3 mg/day	
Sample size	342	114	114	114	
Model 1 β (95% CI)[1]	-0.11 (-0.29,0.07)	0	-0.18 (-0.68,0.32)	-0.47 (-0.98,0.03)	0.06
Model 2 β (95% CI)[2]	-0.13 (-0.32,0.06)	0	-0.19 (-0.69,0.31)	**-0.52 (-1.04,-0.01)**	**0.04**
Model 3 β (95% CI)[3]	0.00 (-0.21,0.22)	0	-0.04 (-0.57,0.49)	-0.19 (-0.82,0.45)	0.55
T2DM	SD/day	<2.3 mg/day	2.3-3.2 mg/day	>3.2 mg/day	
Sample size	586	195	196	195	
Model 1 β (95% CI)[1]	-0.06 (-0.21,0.09)	0	-0.13 (-0.54,0.27)	-0.36 (-0.77,0.05)	0.08
Model 2 β (95% CI)[2]	-0.08 (-0.22,0.07)	0	-0.11 (-0.51,0.30)	-0.35 (-0.76,0.07)	0.10
Model 3 β (95% CI)[3]	0.03 (-0.15,0.21)	0	0.06 (-0.37,0.50)	-0.05 (-0.58,0.48)	0.83
MG-H1					
Normal glucose metabolism	SD/day	<20.5 mg/day	20.5-26.7 mg/day	>26.7 mg/day	
Sample size	1327	442	443	442	
Model 1 β (95% CI)[1]	-0.01 (-0.10,0.08)	0	-0.09 (-0.29,0.13)	-0.01 (-0.20,0.19)	0.97
Model 2 β (95% CI)[2]	0.00 (-0.09,0.09)	0	-0.08 (-0.27,0.11)	0.02 (-0.18,0.22)	0.76
Model 3 β (95% CI)[3]	-0.03 (-0.16,0.11)	0	-0.09 (-0.30,0.11)	-0.00 (-0.27,0.26)	0.93
Prediabetes	SD/day	<19.9 mg/day	19.9-26.7 mg/day	>26.7 mg/day	
Sample size	342	114	114	114	
Model 1 β (95% CI)[1]	-0.11 (-0.28,0.07)	0	-0.25 (-0.75,0.25)	-0.46 (-0.96,0.04)	0.08
Model 2 β (95% CI)[2]	-0.11 (-0.28,0.07)	0	-0.21 (-0.72,0.29)	-0.42 (-0.92,0.09)	0.11
Model 3 β (95% CI)[3]	0.04 (-0.17,0.26)	0	-0.00 (-0.55,0.55)	0.06 (-0.64,0.75)	0.87
T2DM	SD/day	<19.5 mg/day	19.5-25.9 mg/day	>25.9 mg/day	
Sample size	586	195	196	195	
Model 1 β (95% CI)[1]	-0.11 (-0.26,0.05)	0	0.09 (-0.31,0.50)	**-0.41 (-0.81,-0.00)**	**0.03**
Model 2 β (95% CI)[2]	-0.12 (-0.27,0.04)	0	0.06 (-0.35,0.47)	**-0.42 (-0.83,-0.02)**	**0.03**
Model 3 β (95% CI)[3]	0.01 (-0.20,0.22)	0	0.18 (-0.26,0.62)	-0.20 (-0.73,0.33)	0.39

Abbreviations: AGE, advanced glycation endproduct; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Regression coefficients (B) and 95% CI represent the difference in cfPWV (in m/s) per 1-SD change in dietary AGE intake or for a dietary AGE tertile compared to the reference tertile while adjusted for age, sex and heart rate and mean arterial pressure obtained during vascular measurements.

[2] Additionally adjusted for waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, and kidney function.

[3] Additionally adjusted for energy intake, educational level, physical activity and the Dutch Healthy Diet index.

Supplementary Table 3.5 Associations between dietary AGEs and arterial stiffness in 1128 apparently healthy adults of The Maastricht Study

	Continuous *n* = 1128	**Tertile 1** (Ref) *n* = 376	**Tertile 2** *n* = 376	**Tertile 3** *n* = 376	P_{trend}
cfPWV (m/s)					
CML	SD/day	<2.7 mg/day	2.7-3.6 mg/day	>3.6 mg/day	
Model 1 β (95% CI)[1]	-0.02 (-0.12,0.07)	0	0.08 (-0.13,0.29)	0.00 (-0.22,0.22)	0.95
Model 2 β (95% CI)[2]	-0.02 (-0.11,0.08)	0	0.08 (-0.13,0.29)	0.01 (-0.21,0.23)	0.97
Model 3 β (95% CI)[3]	0.04 (-0.11,0.19)	0	0.14 (-0.09,0.36)	0.14 (-0.16,0.44)	0.40
CEL	SD/day	<2.4 mg/day	2.4-3.2 mg/day	>3.2 mg/day	
Model 1 β (95% CI)[1]	0.03 (-0.06,0.13)	0	0.03 (-0.18,0.24)	-0.05 (-0.26,0.16)	0.60
Model 2 β (95% CI)[2]	0.03 (-0.07,0.13)	0	0.02 (-0.19,0.22)	-0.07 (-0.28,0.15)	0.51
Model 3 β (95% CI)[3]	0.09 (-0.04,0.22)	0	0.04 (-0.18,0.26)	-0.01 (-0.28,0.26)	0.90
MG-H1	SD/day	<20.4 mg/day	20.4-26.6 mg/day	>26.6 mg/day	
Model 1 β (95% CI)[1]	-0.04 (-0.14,0.06)	0	-0.07 (-0.28,0.14)	-0.06 (-0.27,0.16)	0.65
Model 2 β (95% CI)[2]	-0.03 (-0.13,0.07)	0	-0.07 (-0.28,0.13)	-0.04 (-0.26,0.17)	0.74
Model 3 β (95% CI)[3]	-0.00 (-0.15,0.14)	0	-0.03 (-0.26,0.19)	0.04 (-0.25,0.33)	0.75
carDC (mm^2/kPa)					
CML	SD/day	<2.7 mg/day	2.7-3.6 mg/day	>3.6 mg/day	
Model 1 β (95% CI)[1]	**-0.31 (-0.58,-0.03)**	0	0.43 (-0.17,1.03)	**-0.63 (-1.25,-0.01)**	**0.03**
Model 2 β (95% CI)[2]	**-0.29 (-0.56,-0.02)**	0	0.46 (-0.13,1.06)	-0.57 (-1.20,0.05)	**0.04**
Model 3 β (95% CI)[3]	-0.04 (-0.48,0.40)	0	0.67 (0.02,1.32)	-0.11 (-0.98,0.75)	0.64
CEL	SD/day	<2.4 mg/day	2.4-3.2 mg/day	>3.2 mg/day	
Model 1 β (95% CI)[1]	-0.17 (-0.45,0.11)	0	-0.01 (-0.60,0.59)	-0.09 (-0.71,0.52)	0.75
Model 2 β (95% CI)[2]	-0.12 (-0.41,0.16)	0	0.05 (-0.55,0.64)	0.02 (-0.59,0.64)	0.95
Model 3 β (95% CI)[3]	0.18 (-0.19,0.54)	0	0.36 (-0.27,0.98)	0.74 (-0.02,1.50)	0.06
MG-H1	SD/day	<20.4 mg/day	20.4-26.6 mg/day	>26.6 mg/day	
Model 1 β (95% CI)[1]	**-0.34 (-0.62,-0.06)**	0	-0.01 (-0.60,0.59)	-0.44 (-1.05,0.18)	0.14
Model 2 β (95% CI)[2]	**-0.33 (-0.61,-0.05)**	0	-0.00 (-0.59,0.59)	-0.41 (-1.03,0.20)	0.17
Model 3 β (95% CI)[3]	-0.08 (-0.49,0.34)	0	0.33 (-0.31,0.97)	0.30 (-0.52,1.12)	0.51
carYEM (10^3/kPa)					
CML	SD/day	<2.7 mg/day	2.7-3.6 mg/day	>3.6 mg/day	
Model 1 β (95% CI)[1]	0.02 (-0.00,0.03)	0	-0.02 (-0.06,0.02)	**0.04 (0.01,0.08)**	**0.03**
Model 2 β (95% CI)[2]	0.02 (-0.00,0.03)	0	-0.02 (-0.06,0.02)	0.04 (-0.00,0.08)	**0.04**
Model 3 β (95% CI)[3]	-0.01 (-0.04,0.02)	0	-0.04 (-0.08,0.01)	0.00 (-0.05,0.06)	0.80
CEL	SD/day	<2.4 mg/day	2.4-3.2 mg/day	>3.2 mg/day	
Model 1 β (95% CI)[1]	0.01 (-0.01,0.03)	0	0.01 (-0.03,0.05)	0.01 (-0.03,0.05)	0.63
Model 2 β (95% CI)[2]	0.00 (-0.02,0.02)	0	0.00 (-0.04,0.04)	0.00 (-0.04,0.04)	0.96
Model 3 β (95% CI)[3]	-0.02 (-0.04,0.00)	0	-0.02 (-0.06,0.02)	-0.05 (-0.10,0.00)	0.06
MG-H1	SD/day	<20.4 mg/day	20.4-26.6 mg/day	>26.6 mg/day	
Model 1 β (95% CI)[1]	0.02 (-0.00,0.03)	0	-0.02 (-0.05,0.02)	0.02 (-0.02,0.06)	0.20
Model 2 β (95% CI)[2]	0.02 (-0.00,0.03)	0	-0.01 (-0.05,0.02)	0.02 (-0.02,0.06)	0.22
Model 3 β (95% CI)[3]	-0.01 (-0.03,0.02)	0	-0.04 (-0.08,0.02)	-0.03 (-0.08,0.03)	0.43

Abbreviations: AGE, advanced glycation endproduct; CarDC, carotid distension coefficient; CarYEM, carotid Young's Elastic Modulus; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Regression coefficients (Beta) and 95% CI represent the difference in arterial stiffness (in m/s, for cfPWV, mm^2/kPa for carDC, and 10^3/kPa for carYEM) per 1-SD change in dietary AGE intake or for a dietary AGE tertile compared to the reference tertile while adjusted for age, sex, glucose metabolism status, and heart rate and mean arterial pressure obtained during vascular measurements.

[2] Additionally adjusted for waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, and kidney function.

[3] Additionally adjusted for energy intake, educational level, physical activity and the Dutch Healthy Diet index.

Supplementary Table 3.6 Associations between dietary AGEs and carotid YEM in 1912 adults of The Maastricht Study while substituting physical activity obtained from questionnaires for accelerometer data.

Dietary AGE	**Continuous** *n* = 1912	**Quartile 1** (ref) *n* = 478	**Quartile 2** *n* = 478	**Quartile 3** *n* = 478	**Quartile 4** *n* = 478	P_{trend}
CML	SD/day	<2.5 mg/day	2.5-3.1 mg/day	3.1-3.9 mg/day	>3.9 mg/day	
Model 1 β (95% CI)[1]	0.01 (-0.01,0.02)	0	-0.01 (-0.04,0.03)	0.01 (-0.02,0.05)	0.02 (-0.02,0.06)	0.26
Model 2 β (95% CI)[2]	0.01 (-0.01,0.02)	0	-0.01 (-0.04,0.03)	0.01 (-0.02,0.05)	0.02 (-0.02,0.05)	0.27
Model 3 β (95% CI)[3]	0.01 (-0.01,0,03)	0	-0.01 (-0.04,0.03)	0.02 (-0.03,0.06)	0.02 (-0.04,0.07)	0.39
CEL	SD/day	<2.2 mg/day	2.2-2.8 mg/day	2.8-3.5 mg/day	>3.5 mg/day	
Model 1 β (95% CI)[1]	0.00 (-0.01,0.02)	0	-0.02 (-0.06,0.01)	-0.02 (-0.06,0.02)	-0.02 (-0.06,0.02)	0.50
Model 2 β (95% CI)[2]	0.00 (-0.01,0.02)	0	-0.02 (-0.06,0.02)	-0.02 (-0.05,0.02)	-0.01 (-0.05,0.02)	0.60
Model 3 β (95% CI)[3]	-0.00 (-0.02,0.02)	0	-0.03 (-0.06,0.01)	-0.03 (-0.07,0.01)	-0.03 (-0.08,0.01)	0.24
MG-H1	SD/day	<18.6 mg/day	18.6-23.0 mg/day	23.0-28.5 mg/day	>28.5 mg/day	
Model 1 β (95% CI)[1]	0.01 (-0.01,0.02)	0	-0.01 (-0.05,0.02)	0.02 (-0.02,0.06)	0.00 (-0.03,0.04)	0.56
Model 2 β (95% CI)[2]	0.01 (-0.01,0.03)	0	-0.01 (-0.05,0.03)	0.02 (-0.02,0.06)	0.01 (-0.03,0.05)	0.42
Model 3 β (95% CI)[3]	0.01 (-0.01,0.03)	0	-0.01 (-0.05,0.03)	0.02 (-0.02,0.06)	0.01 (-0.05,0.06)	0.64

Abbreviations: AGE, advanced glycation endproduct; Carotid YEM, carotid Young's Elastic Modulus; CEL, N^{ε}-(1-carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Regression coefficients (β) and 95% CI represent the difference in carYEM (10^3/kPa) per SD-change in dietary AGE intake or for categorical analyses, difference for a dietary AGE quartile compared to the reference quartile while adjusted for age, sex, glucose metabolism status and heart rate and mean arterial pressure obtained during vascular measurements.

[2] Additionally adjusted for waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, and kidney function.

[3] Additionally adjusted for energy intake, educational level, physical activity and the Dutch Healthy Diet index.

Supplementary Table 3.7 Associations between dietary AGEs and cfPWV in adults of The Maastricht Study, while substituting physical activity obtained from questionnaires for accelerometer data, stratified for glucose metabolism status.

Dietary AGE	Continuous	Tertile 1 (ref)	Tertile 2	Tertile 3	*P* trend
CML					
Total group	SD/day	<2.7 mg/day	2.7-3.6 mg/day	>3.6 mg/day	
Sample size	1912	637	637	638	
Model 1 β (95% CI)[1]	-0.08 (-0.16, 0.01)	0	-0.06 (-0.25,0.13)	-0.19 (-0.39,0.00)	**0.05**
Model 2 β (95% CI)[2]	-0.08 (-0.16,0.00)	0	-0.06 (-0.25,0.13)	-0.19 (-0.38,0.01)	0.06
Model 3 β (95% CI)[3]	0.01 (-0.11,0.14)	0	0.02 (-0.19,0.23)	-0.01 (-0.28,0.26)	0.93
Normal glucose metabolism	SD/day	<2.7 mg/day	2.7-3.6 mg/day	>3.6 mg/day	
Sample size	1082	360	361	361	
Model 1 β (95% CI)[1]	-0.01 (-0.11,0.10)	0	0.18 (-0.06,0.41)	0.12 (-0.12,0.37)	0.68
Model 2 β (95% CI)[2]	0.00 (-0.10,0.10)	0	0.13 (-0.08,0.34)	0.07 (-0.15,0.29)	0.59
Model 3 β (95% CI)[3]	0.05 (-0.12,0.21)	0	0.16 (-0.07,0.39)	0.15 (-0.16,0.45)	0.39
Prediabetes	SD/day	<2.8 mg/day	2.8-3.7 mg/day	>3.7 mg/day	
Sample size	278	95	97	95	
Model 1 β (95% CI)[1]	-0.16 (-0.39,0.07)	0	-0.11 (-0.68,0.46)	-0.31 (-0.89,0.26)	0.28
Model 2 β (95% CI)[2]	-0.17 (-0.40,0.06)	0	-0.05 (-0.64,0.54)	-0.31 (-0.89,0.28)	0.30
Model 3 β (95% CI)[3]	-0.03 (-0.35,0.29)	0	0.20 (-0.44,0.85)	0.22 (-0.57,1.01)	0.60
T2DM	SD/day	<2.7 mg/day	2.7-3.6 mg/day	>3.6 mg/day	
Sample size	543	181	181	181	
Model 1 β (95% CI)[1]	-0.11 (-0.27,0.06)	0	0.34 (-0.76,0.08)	**-0.46 (-0.89,-0.03)**	**0.05**
Model 2 β (95% CI)[2]	-0.12 (-0.28,0.05)	0	-0.35 (-0.77,0.08)	-0.47 (0.90,-0.04)	**0.04**
Model 3 β (95% CI)[3]	0.01 (-0.24,0.27)	0	-0.25 (-0.71,0.22)	-0.34 (-0.94,0.26)	0.28
CEL					
Total group	SD/day	<2.4 mg/day	2.4-3.2 mg/day	>3.2 mg/day	
Sample size	1912	637	637	638	
Model 1 β (95% CI)[1]	-0.04 (-0.12,0.04)	0	-0.02 (-0.21,0.17)	**-0.27 (-0.51,-0.08)**	**0.00**
Model 2 β (95% CI)[2]	-0.05 (-0.13,0.04)	0	-0.03 (-0.22,0.16)	**-0.28 (-0.47,-0.08)**	**0.00**
Model 3 β (95% CI)[3]	0.03 (-0.07,0.13)	0	0.02 (-0.18,0.22)	-0.18 (-0.42,0.06)	0.11
Normal glucose metabolism	SD/day	<2.4 mg/day	2.4-3.4 mg/day	>3.2 mg/day	
Sample size	1082	361	361	360	
Model 1 β (95% CI)[1]	0.04 (-0.06,0.15)	0	0.09 (-0.13,0.29)	-0.00 (-0.22,0.21)	0.92
Model 2 β (95% CI)[2]	0.04 (-0.07,0.14)	0	0.07 (-0.14,0.28)	-0.02 (-0.24,0.20)	0.80
Model 3 β (95% CI)[3]	0.09 (-0.05,0.23)	0	0.07 (-0.16,0.29)	-0.03 (-0.30,0.25)	0.81
Prediabetes	SD/day	<2.4 mg/day	2.4-3.3 mg/day	>3.3 mg/day	
Sample size	278	96	95	96	
Model 1 β (95% CI)[1]	-0.09 (-0.29,0.12)	0	-0.06 (-0.63,0.50)	-0.49 (-1.05,0.08)	0.08
Model 2 β (95% CI)[2]	-0.09 (-0.30,0.12)	0	-0.08 (-0.66,0.50)	-0.50 (-1.08,0.08)	0.08
Model 3 β (95% CI)[3]	0.01 (-0.23,0.25)	0	0.06 (-0.55,0.68)	-0.21 (-0.92,0.49)	0.50
T2DM	SD/day	<2.3 mg/day	2.3-3.2 mg/day	>3.2 mg/day	
Sample size	543	181	181	181	
Model 1 β (95% CI)[1]	-0.07 (-0.22,0.09)	0	-0.21 (-0.63,0.21)	**-0.56 (-0.98,-0.13)**	**0.01**
Model 2 β (95% CI)[2]	-0.08 (-0.24,0.08)	0	-0.17 (-0.59,0.25)	**-0.54 (-0.97,-0.11)**	**0.01**
Model 3 β (95% CI)[3]	0.00 (-0.18,0.19)	0	-0.10 (-0.55,0.35)	-0.44 (-0.98,0.10)	0.10
MG-H1					
Total group	SD/day	<20.1 mg/day	20.1-26.3 mg/day	>26.3 mg/day	
Sample size	1912	637	637	638	
Model 1 β (95% CI)[1]	**-0.10 (-0.18,-0.01)**	0	-0.12 (-0.31,0.07)	**-0.32 (-0.54,-0.11)**	**<0.01**
Model 2 β (95% CI)[2]	**-0.09 (-0.17,-0.01)**	0	-0.11 (-0.31,0.08)	**-0.29 (-0.51,-0.13)**	**<0.01**
Model 3 β (95% CI)[3]	-0.02 (-0.13,0.09)	0	-0.08 (-0.28,0.12)	-0.20 (-0.46,0.06)	0.12
Normal glucose metabolism	SD/day	<20.5 mg/day	20.5-26.7 mg/day	>26.7 mg/day	
Sample size	1082	360	361	361	
Model 1 β (95% CI)[1]	-0.06 (-0.16,0.04)	0	-0.08 (-0.28,0.14)	-0.05 (-0.26,0.17)	0.68
Model 2 β (95% CI)[2]	-0.05 (-0.15,0.06)	0	-0.07 (-0.28,0.14)	-0.02 (-0.24,0.19)	0.86
Model 3 β (95% CI)[3]	-0.08 (-0.23,0.08)	0	-0.08 (-0.31,0.15)	-0.04 (-0.33,0.25)	0.86
Prediabetes	SD/day	<19.9 mg/day	19.9-26.7 mg/day	>26.7 mg/day	
Sample size	278	95	96	96	
Model 1 β (95% CI)[1]	-0.06 (-0.26,0.13)	0	-0.09 (-0.66,0.48)	-0.37 (-0.94,0.20)	0.19
Model 2 β (95% CI)[2]	-0.06 (-0.26,0.14)	0	-0.05 (-0.63,0.54)	-0.36 (-0.94,0.22)	0.21
Model 3 β (95% CI)[3]	0.06 (-0.18,0.30)	0	0.16 (-0.47,0.79)	0.10 (-0.69,0.88)	0.84
T2DM	SD/day	<19.5 mg/day	19.5-25.9 mg/day	>25.9 mg/day	
Sample size	543	181	181	181	
Model 1 β (95% CI)[1]	-0.12 (-0.29,0.05)	0	-0.11 (-0.53,0.31)	**-0.63 (-1.05,-0.21)**	**<0.01**
Model 2 β (95% CI)[2]	-0.13 (-0.30,0.04)	0	-0.09 (-0.52,0.33)	**-0.62 (-1.04,-0.20)**	**<0.01**
Model 3 β (95% CI)[3]	-0.04 (-0.26,0.19)	0	-0.08 (-0.53,0.38)	**-0.60 (-1.15,-0.05)**	**0.02**

Abbreviations: AGE, advanced glycation endproduct; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.
[1] Regression coefficients (Beta) and 95% CI represent the difference in cfPWV (in m/s) per 1-SD change in dietary AGE intake and per change in dietary AGE tertile compared to the reference tertile while adjusted for age, sex, and heart rate and mean arterial pressure obtained during vascular measurements. [2] Additionally adjusted for waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, and kidney function. [3] Additionally adjusted for energy intake, educational level, physical activity, and the Dutch Healthy Diet index.

Supplementary Table 3.8 Associations between dietary AGEs from food groups and cfPWV (m/s) in 2255 adults of The Maastricht Study.

AGEs from food groups β (95% CI) per 1 SD/day[1]	CML	CEL	MG-H1
Cereals	-0.03 (-0.11,0.05)	-0.01 (-0.07,0.09)	-0.01 (-0.07,0.09)
Meat	0.06 (-0.02,0.14)	0.06 (-0.02,0.13)	0.03 (-0.05,0.10)
Fish	-0.05 (-0.12,0.03)	-0.04 (-0.12,0.03)	-0.04 (-0.12,0.03)
Dairy	0.04 (-0.03,0.11)	0.03 (-0.05,0.10)	0.02 (-0.05,0.10)
Confectionaries	-0.05 (-0.12,0.03)	-0.06 (-0.14,0.01)	-0.07 (-0.14,0.01)
Nuts	0.05 (-0.02,0.13)	0.05 (-0.02,0.13)	0.05 (-0.02,0.13)

Abbreviations: AGE, advanced glycation endproduct; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Regression coefficients (β) and 95% CI represent the difference in cfPWV (m/s) per SD-change in dietary AGE intake while adjusted for age, sex, and glucose metabolism status, heart rate and mean arterial pressure obtained during vascular measurements, waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, and kidney function, energy intake, educational level, physical activity, and the Dutch Healthy Diet index.

Supplementary Table 3.9 Associations between dietary AGEs from food groups and carotid YEM (10^3/kPa) in 2255 adults of The Maastricht Study.

AGEs from food groups β (95% CI) per 1 SD/day[1]	CML	CEL	MG-H1
Cereals	0.01 (-0.00,0.03)	0.01 (-0.01,0.02)	0.01 (-0.01,0.02)
Meat	-0.01 (-0.02,0.01)	-0.01 (-0.02,0.01)	0.00 (-0.01,0.02)
Fish	-0.00 (-0.02,0.01)	-0.01 (-0.02,0.01)	-0.00 (-0.02,0.01)
Dairy	0.00 (-0.01,0.02)	0.00 (-0.01,0.02)	0.00 (-0.01,0.01)
Confectionaries	-0.00 (-0.02,0.01)	-0.00 (-0.01,0.01)	0.00 (-0.01,0.01)
Nuts	-0.01 (-0.02,0.00)	-0.01 (-0.02,0.00)	-0.01 (-0.02,0.00)

Abbreviations: AGE, advanced glycation endproduct; Carotid YEM, carotid Young's Elastic Modulus; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Regression coefficients (β) and 95% CI represent the difference in carotid YEM (10^3/kPa) per SD-change in dietary AGE intake while adjusted for age, sex, and glucose metabolism status, heart rate and mean arterial pressure obtained during vascular measurements, waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, and kidney function, energy intake, educational level, physical activity, and the Dutch Healthy Diet index.

Supplementary Table 3.10 Associations between dietary AGEs from food groups and carotid DC (mm^2/kPa) in 2255 adults of The Maastricht Study.

AGEs from food groups β (95% CI) per 1 SD/day[1]	CML	CEL	MG-H1
Cereals	**-0.27 (-0.47,-0.07)**	**-0.23 (-0.42,-0.04)**	-0.17 (-0.37,0.03)
Meat	-0.02 (-0.22,0.18)	-0.01 (-0.19,0.17)	-0.11 (-0.28,0.07)
Fish	0.01 (-0.17,0.19)	0.03 (-0.15,0.21)	0.00 (-0.18,0.18)
Dairy	-0.06 (-0.24,0.12)	-0.06 (-0.25,0.13)	-0.10 (-0.29,0.08)
Confectionaries	0.18 (-0.01,0.36)	0.14 (-0.04,0.32)	0.12 (-0.06,0.30)
Nuts	0.06 (-0.13,0.24)	0.06 (-0.13,0.24)	0.06 (-0.13,0.24)

Abbreviations: AGE, advanced glycation endproduct; Carotid DC, carotid distension coefficient; CEL, N^{ε}-(1-carboxyethyl)lysine; cfPWV, carotid-to-femoral pulse wave velocity; CML, N^{ε}-(carboxymethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

[1] Regression coefficients (β) and 95% CI represent the difference in carotid DC (mm^2/kPa) per SD-change in dietary AGE intake while adjusted for age, sex, and glucose metabolism status, heart rate and mean arterial pressure obtained during vascular measurements, waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, use of antihypertensive medication, prior CVD, alcohol intake, and kidney function, energy intake, educational level, physical activity, and the Dutch Healthy Diet index.

dietary AGEs in TWIX

CML: 0.9; CEL 0.8; MG-H1: 5.8 mg/100g

Chapter 4

Habitual intake of dietary advanced glycation endproducts is not associated with generalized microvascular function - The Maastricht Study

Armand M.A. Linkens, Alfons J.H.M. Houben, Abraham A. Kroon, Miranda T. Schram, Tos T.J.M. Berendschot, Carroll A.B. Webers, Marleen van Greevenbroek, Ronald M.A. Henry, Bastiaan de Galan, Coen D.A. Stehouwer, Simone J.M.P. Eussen, Casper G. Schalkwijk.

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Abstract

Background: Endogenously-formed advanced glycation endproducts (AGEs) may be important drivers of microvascular dysfunction and the microvascular complications of diabetes. AGEs are also formed in food products, especially during preparation methods involving dry heat.

Objective: To assess cross-sectional associations between dietary AGE intake and generalized microvascular function in a population-based cohort.

Methods: In 3140 participants of The Maastricht Study (age 60 ± 8 years, 51% men) dietary AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) were estimated using the combination of our ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) dietary AGE database and a food frequency questionnaire (FFQ). Microvascular function was determined in the retina as flicker light-induced arteriolar and venular dilation and as central arteriolar and venular equivalent (CRAE and CRVE), in plasma as a z-score of endothelial dysfunction biomarkers (soluble vascular and intracellular adhesion molecules 1 (sVCAM-1 and sICAM-1), soluble eSelectin, and von Willebrand Factor (vWf)), in skin as the heat-induced skin hyperemic response, and in urine as 24-hour albuminuria. Associations were evaluated using multiple linear regression adjusting for demographical, cardiovascular, lifestyle, and dietary factors.

Results: Overall, intake of CML, CEL, and MG-H1 were not associated with the microvascular outcomes. Although higher intake of CEL was associated with higher flicker light-induced venular dilation (beta %-change over baseline (95% CI) of 0.14 (0.02,0.26)) and lower plasma biomarker z-score (beta SD (95% CI) of -0.04 (-0.08,-0.00)), the effect sizes were small and their biological relevance can be questioned.

Conclusions: We did not show any strong association between habitual intake of dietary AGEs and generalized microvascular function. The contribution of dietary AGEs to generalized microvascular function should be further assessed in randomized controlled trials using specifically-designed dietary interventions.

Introduction

Through a wide range of biological effects, advanced glycation endproducts (AGEs) may be important drivers of microvascular dysfunction[1, 2], which in turn is an important risk factor for microvascular diseases such as retinopathy, nephropathy, and neuropathy[3]. Endogenous formation of AGEs, a heterogeneous group of sugar-modified proteins[4], is increased under conditions of chronic hyperglycemia and increased oxidative stress[5].

Aside from endogenous formation, AGEs are also formed during preparation of foods, especially in products such as heat-treated cereals and meats[6]. We recently found in humans that dietary AGEs are associated with their levels in plasma, suggesting their absorption[7]. Whether these dietary AGEs also contribute to microvascular function, and what mechanisms are involved, is currently unknown. Studies investigating the effects of dietary AGEs on microvascular function are scarce and mainly limited to plasma biomarkers of endothelial dysfunction. A recent meta-analysis of intervention trials showed that a low AGE diet, compared to a high AGE diet, reduces soluble vascular adhesion molecule 1 (sVCAM-1) levels[8]. However, as microvascular function differs among tissues, measuring plasma biomarkers alone provides limited insight into general microvascular function[9]. While these plasma biomarkers reflect endothelial regulation of permeability, coagulation, fibrinolysis, and proliferation, microvascular function can also be assessed by the endothelium-dependent vasodilation response to various stimuli, such as local heating of the skin and flicker light applied to the retina[9]. One study investigating the effect of a low or high AGE diet for 6 weeks found no differences in endothelial function (i.e. reactive hyperemia index)[10]. However, most of these studies share common methodological limitations, namely relatively small sample sizes, short study durations, extreme differences in cooking methods to modulate dietary AGEs, and the usage of an immunohistochemistry-based database that contains only one dietary AGE, N^{ε}-(carboxymethyl)lysine (CML). Several other AGEs are present in foods, including N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), which potentially have different biological effects. Because these AGEs are formed on different amino acids (CML and CEL from lysine, and MG-H1 from arginine), their intake may vary depending on an individual's dietary pattern. As such, the role of specific dietary AGEs in general microvascular function remains to be established.

In light of the above, we investigated the association between habitual intake of the specific and well-characterized dietary AGEs CML, CEL, and MG-H1, and generalized microvascular function measured in the retina, skin, blood, and kidney in a large population-based cohort.

Material and Methods

Study design and population

We used data from The Maastricht Study, an observational prospective population-based cohort study. The rationale and methodology have been described previously[11]. In brief, the study focuses on the etiology, pathophysiology, complications and comorbidities of type 2 diabetes mellitus (T2DM) and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Participants were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. Recruitment was stratified according to known T2DM status, with an oversampling of individuals with T2DM, for reasons of efficiency. The present report includes cross-sectional data from the first 3451 participants, who completed the baseline survey between November 2010 and September 2013. The examinations of each participant were performed within a time window of three months. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent. Primary outcomes of this study were measurements of microvascular function at several microvascular beds. In the retina, microvascular function was determined as flicker light-induced arteriolar and venular dilation and as central arteriolar and venular equivalent. In plasma, endothelial dysfunction was determined by endothelial dysfunction biomarkers (soluble vascular and intracellular adhesion molecule 1 [VCAM-1 and sICAM-1], soluble eSelectin, and von Willebrand Factor [vWf]). In skin, this was determined as the heat-induced skin hyperemic response. Finally, in urine, this was determined as 24-hour albuminuria.

Food intake and dietary advanced glycation endproducts

We assessed dietary intake by a validated 253-item food frequency questionnaire (FFQ)[12]. This FFQ contains 101 questions on consumption with a reference period of one year. The FFQ collected information on the intake of major food groups. All participants filled out the FFQ after their first visit to the study center.

Food intake was determined by the combination of frequency questions with quantity questions. For the frequency questions, 11 options were available ranging from “not used” to 7 days/week. For the quantity questions, variable options were available based on fourteen standard household servings, ranging from < 1/day to > 12/day. Average daily consumption of food items was then calculated by multiplying the

frequency and amount. Energy and nutrient intakes were subsequently determined by transcribing food items into food codes embedded in the Dutch Food Composition Table 2011[13]. Additionally, we determined the Dutch Healthy Diet (DHD) index based on this food intake data. The DHD-index is a measure of diet quality as it assesses adherence to the Dutch dietary guidelines[14]. A higher index has been associated with more nutrient-dense diets and lower risk of mortality[15, 16]. In our statistical analyses we used a modified version of the DHD-index that does not contain filtered coffee consumption as this information was not collected by the FFQ, and alcohol intake was not included as we already adjusted for alcohol intake as an individual variable.

Dietary AGE intake was determined by coupling the consumption of food items within the FFQ to our dietary AGE database[6]. In this database, three major AGEs, CML, CEL, and MG-H1, were quantified in protein fractions of food products using highly specific ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). In total, this database includes over 200 food products commonly consumed in a Western diet. For each participant, AGE intake was estimated as described previously[7]. Some of the food products in the FFQ were not analyzed for AGEs content. AGE content of these specific products were estimated by matching them to other products that were comparable in macronutrient profile and preparation method. For example, for several fresh vegetables boiled in water, such as endive, beets, leek, and spinach, the same AGE content was used. By comparison, jarred peas and carrots were measured separately from fresh peas and carrots, as AGEs in jarred peas and carrots are higher as they contain added sugar and are heated to prolong shelf life[6]. Only after completion of the FFQ, participants were informed about their glucose metabolism status. As a result, estimation of dietary AGEs was not affected by a potential change in dietary habits upon dietary advice from health care workers in case participants were diagnosed with T2DM.

Assessment of microvascular function

All participants were asked to refrain from smoking and drinking caffeine-containing beverages 3 hours before the measurement. A light meal (breakfast or lunch), low in fat content, was allowed at least 90 minutes before the start of the measurements. For these microvascular measurements there is no international consensus on how to perform them in a standardized manner. Protocols for the Maastricht Study were designed based on our experience and best practice in other studies[9]. All microvascular measurements have been extensively validated as described previously[17].

Retinal arteriolar and venular dilation response

The retinal vascular response to flicker light was assessed with the Dynamic Vessel Analyzer (DVA, Imedos, Jena, Germany) as described previously[18]. Briefly, pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine at least 15min prior to the start of the examination. For safety reasons, participants with an intraocular pressure more than 30mmHg were excluded from the measurements. For each participant, either the left or the right eye was selected depending on the time of day the measurement was performed and without reference to participant characteristics. A straight arteriolar or venular segment of approximately 1.5mm in length located 0.5–2 disc diameter from the margin of the optic disc in the temporal section was examined. Vessel diameter was automatically and continuously measured for 150s. A baseline recording of 50s was followed by a 40s flicker light exposure period (flicker frequency 12.5Hz, bright-to-dark contrast ratio 25 : 1), followed by a 60s recovery period. Baseline retinal vascular diameters and flicker light-induced retinal vascular dilation were automatically calculated with the integrated DVA software (version 4.51; Imedos, Jena, Germany). Baseline retinal arteriolar/venular diameter was calculated as the average diameter of the 20–50s recording and was expressed in measurement units (MU), where one measurement unit is equal to 1 mm of the Gullstrand eye.

The flickerlight-induced retinal vascular dilation was expressed as the percentage retinal vascular dilation over baseline and based on the average dilation achieved at time-points 10 and 40s during the flicker stimulation period. This dilation response depends on a process called neurovascular coupling, which involves endothelial function[17].

Static retinal vessel calibers

All fundus photographs were taken with an auto-focus, auto-shot and auto-tracking fundus camera (Model AFC-230; Nidek, Gamagori, Japan) in an optic disc-centered field of view of 45° in a darkened room, as described previously[19]. Static retinal vessel analysis (one image of the left or right eye was randomly chosen for each participant) was performed using the RHINO software (Eindhoven, the Netherlands)[20, 21]. Optic disc detection and arteriole/venule classification were corrected manually. Retinal vessel diameters were measured at 0.5-1.0 disc diameter away from the optic disc margin and were presented as central retinal arteriolar equivalent and central retinal venular equivalent (CRAE and CRVE, respectively) in MU. The scale factor is based on the optic disc diameter, which is assumed to be 1800 µm 22, i.e. 1 MU =1 pixelsize×1800 µm/pixelsize of optic disc

diameter. CRAE and CRVE represent the equivalent single-vessel parent diameter for the six largest arterioles and largest venules in the region of interest, respectively. The calculations were based on the improved Knudtson–Parr–Hubbard formula[23]. Fundus photographs of insufficient quality, e.g. obstructed by lashes or defocused, were evaluated and discussed with a second observer and excluded on mutual agreement. Narrowing of retinal arterioles and widening of retinal venules are regarded as early indicators of cardiovascular disease[24].

Plasma biomarkers of endothelial dysfunction

The plasma biomarkers of endothelial dysfunction sVCAM-1, sICAM-1, and sE-selectin were measured in EDTA plasma samples with commercially available 4-plex sandwich immunoassay kits (Meso Scale Discovery (MSD), Rockville, MD, US), as described elsewhere[25]. vWf was determined in citrated plasma with sandwich ELISA (Dako, Glostrup, Denmark). Concentrations of vWf were expressed as a percentage of vWf detected in pooled citrated plasma of healthy volunteers. For the present study, the intra- and inter-assay coefficients of variation were 3.5% and 5.9% for sVCAM-1, 2.5% and 5.3% for sICAM-1, 6.4% and 6.0% for sE-selectin, and 3.2% and 5.4% for vWF.

Skin hyperemic response

Skin blood flow was measured as described previously by means of a laser-Doppler system (Periflux 5000; Perimed, Järfalla, Sweden) equipped with a thermostatic laser-Doppler probe (PF457; Perimed) at the dorsal side of the wrist of the left hand[26]. The laser-Doppler output was recorded for 25 minutes with a sample rate of 32 Hz, which gives semiquantitative assessment of skin blood flow expressed in arbitrary perfusion units. Skin blood flow was first recorded unheated for 2 minutes to serve as a baseline. After the 2 minutes of baseline, the temperature of the probe was rapidly and locally increased to 44°C and was then kept constant until the end of the measurement. The heat-induced skin hyperemic response was expressed as the percentage increase in average perfusion units during the 23-minute heating phase over the average baseline perfusion units. Skin perfusion during a period of local heating is thought to be mainly endothelium-dependent[27, 28] and this method is commonly used as a test of skin microvascular function[9].

Albuminuria

Two 24h urine collections were used to assess urinary albumin excretion. Urinary albumin concentration was measured with a standard immunoturbidimetric assay by an automatic analyzer (due to a change of supplier by the Beckman Synchron LX20, Beckman Coulter Inc., Brea, USA; and the Roche Cobas 6000, F. Hoffmann-La Roche, Basel, Switzerland) and multiplied by collection volume to obtain the 24h urinary albumin excretion. A urinary albumin concentration below the detection limit of the assay (2 mg/l for the Beckman Synchron LX20 and 3 mg/l for the Roche Cobas 6000) was set at 1.5 mg/l before multiplying by collection volume. Only urine collections with a collection time between 20h and 28h were considered valid. If needed, urinary albumin excretion was extrapolated to a 24h excretion. Albuminuria is seen as a risk marker for generalized endothelial dysfunction[29].

Glucose Metabolism Status

To determine glucose metabolism, all participants (except those who used insulin) underwent a standardized 7-point oral glucose tolerance test after an overnight fast. Blood samples were taken at baseline and at 15, 30, 45, 60, 90, and 120 minutes after ingestion of a 75 g glucose drink. For safety reasons, participants with a fasting glucose level above 11.0 mmol/L, as determined by a finger prick, did not undergo the oral glucose tolerance test. For these individuals, fasting glucose level and information about glucose-lowering medication were used to determine glucose metabolism status. Glucose metabolism was classified according to the WHO 2006 criteria into normal glucose tolerance, impaired fasting glucose, impaired glucose tolerance, and T2DM[30]. For this study, we defined having either impaired fasting glucose or impaired glucose tolerance as prediabetes.

Covariates

Smoking status and history of cardiovascular disease (CVD) were assessed by a questionnaire. Smoking status was categorized into never, former and current smoker. Waist circumference, total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), triglycerides, fasting plasma glucose, and glycosylated hemoglobin (HbA1c) were determined as described elsewhere[11]. Estimated glomerular filtration rate (eGFR) was computed with the Chronic Kidney Disease Epidemiology Collaboration formula, using serum creatinine and cystatin C[31]. Information on the use of lipid-modifying, antihypertensive medication, and/or glucose-lowering medication that is generic names, doses and frequencies, were collected during an in person medication interview.

Statistical Methods

Analyses were conducted using SPSS version 25 for Windows (IBM Corporation, Armonk, NY, USA). Baseline characteristics are shown for the total sample, and stratified by a dietary AGE z-score that represents an individuals' overall AGE intake. Because we measured three AGEs in food items which differ in abundance, we first calculated z-scores for CEL, CML and MG-H1, which were then averaged into a single dietary AGE z-score. A z-score for the plasma biomarkers of endothelial dysfunction was calculated similarly. Differences in baseline characteristics between individuals stratified by the dietary AGE z-score were tested by means of an ANOVA or chi-squared test, as appropriate. Likewise, participants who were excluded from the analyses because of missing covariates were compared with the included participants by the same statistical methods.

We performed multiple linear regression to investigate the association between each standardized dietary AGE and the combined dietary AGE z-score and generalized microvascular function expressed as the retinal microvascular dilation response, static retinal vessel calibers, plasma biomarkers of endothelial function, skin microvascular function, and albuminuria (unstandardized). We fitted two regression models. In model 1, we adjusted for age (years), sex (male/female), and glucose metabolism status (NGM, prediabetes, T2DM, and other types of diabetes), the latter due to oversampling of individuals with T2DM in The Maastricht Study. In model 2, we additionally adjusted for cardiovascular risk factors and dietary factors: waist circumference (cm), total/high-density lipoprotein cholesterol ratio, triglycerides (mmol/L), use of lipid-lowering medication (%-yes), office systolic blood pressure, use of anti-hypertensive medication (%-yes), smoking status (former, current, never), total energy intake (kcal/day), educational level (low/middle/high), alcohol intake (g/day), and the DHD-index. For the association between dietary AGEs and urinary albumin excretion, model 2 of the regression model was additionally adjusted for estimated glomerular filtration rate. To check whether the assumption of linearity for linear regression was met, regression models using dietary AGEs as continuous and categorical exposures were compared with a likelihood ratio test. None of the models gave a significantly better fit of the data using dietary AGEs as categorical exposures (data not shown). Hence, dietary AGEs were entered as continuous exposures in all models. As urinary albumin excretion was positively skewed, a normal distribution was obtained by natural logarithm transformation.

We also performed several sensitivity analyses. Firstly, we introduced further adjustment for covariates reflecting microangiopathy in the regression models: history of cardiovascular disease, eGFR, retinopathy, and albuminuria (except for

analyses in which albuminuria was the outcome). These covariates may introduce overcorrection, as they also reflect microvascular function. Secondly, we introduced further adjustment for physical activity in the regression models (as assessed by accelerometer data (ActivPAL)). As accelerometer data is not available in a relatively large number of participants, performing a complete case analysis with this covariate may result in selection bias. Thirdly, we explored possible confounding by antihypertensive medication through further specification into renin-angiotensin-aldosteron-system (RAAS) inhibitors and other types of antihypertensive medication. Fourthly, we used ambulant 24-hours blood pressure measurements instead of office blood pressure measurements obtained during the vascular measurements. Fifthly, we used body mass index (BMI) instead of waist circumference as a measure of obesity. Additionally, we performed interaction analyses for age, sex, BMI, glucose metabolism status (as dummy variables), and eGFR (thus also adding eGFR as a covariate in the fully adjusted model) by adding interaction terms in our model. The interaction analysis for T2DM and BMI (with substitution of BMI for waist circumference in the regression model) were performed to investigate potential underreporting of individuals with T2DM, overweight or obesity.

Beta coefficients are reported with their 95% confidence intervals and represent the effect on microvascular outcomes per 1 SD higher dietary AGE intake. Unless stated otherwise, beta coefficients represent the fully adjusted model (model 2). *p* values of < 0.05 are considered statistically significant.

Results

Population characteristics

The selection of participants with available data on dietary AGEs, potential confounders, and estimates of microvascular function are shown in Figure 4.1, and their characteristics are shown in Table 4.1. Mean energy intake was 2182 kcal/day, intake of dietary CML was 3.3 mg/day, of CEL 3.0 mg/day, and of MG-H1 24.3 mg/day, which is comparable to other Dutch cohorts (Supplementary Table 4.1). Participants in the highest dietary AGE quartile were more likely to be younger men, more physically active, consumed more energy and coffee, and had a slightly less healthy diet ($p < 0.05$). These participants had slightly higher systolic and diastolic blood pressure, total/HDL-cholesterol ratio and albuminuria ($p < 0.05$). None of the other microvascular outcomes were statistically different among quartiles of dietary AGE intake. Although the microvascular outcomes could not be assessed in all participants, the selected populations were largely comparable (Supplementary Table 4.2). In general, participants excluded from analyses because of missing variables had a slightly worse cardiovascular risk profile (Supplementary Table 4.3-8).

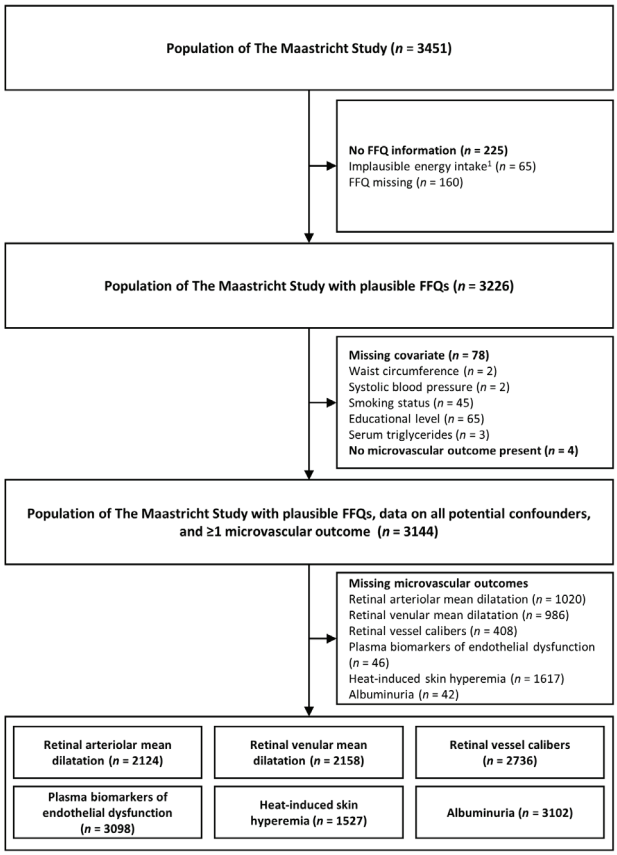

Figure 4.1 Flow chart of participant selection for the different microvascular outcomes. Please note that missing variables do not necessarily add up as they are not mutually exclusive, e.g. an individual may have missing information on both smoking status and educational level.

[1]Implausible energy intake: < 500 kcal or > 3500 kcal for women, < 800 or > 4000 kcal for men.

Abbreviations: FFQ: food frequency questionnaire.

4

Dietary AGEs and retinal microcirculation

Baseline retinal vessel calibers before flicker light were not different between quartiles of dietary AGE intake (data not shown). In the fully adjusted model (model 2), dietary CML, CEL, MG-H1, and the combined dietary AGE z-score were not associated with flicker light-induced retinal arteriolar dilation, with beta (%-change over baseline) (95% CI) of 0.09 (-0.10,0.28), 0.11 (-0.04,0.26), 0.06 (-0.10,0.23), and 0.10 (-0.07,0.28), respectively (Figure 4.2). Greater intake of CEL, but not CML, MG-H1, or the combined dietary AGE z-score was associated with greater flicker light-induced retinal venular dilation: 0.14 (0.02,0.26), 0.14 (-0.02,0.29), 0.07 (-0.07, 0.20), and 0.13 (-0.01,0.27), respectively (Figure 4.2).

In the fully adjusted model, dietary CML, CEL, MG-H1, and the combined dietary AGE z-score were not associated with CRAE (beta (µm) (95% CI) of-0.44 (-1.64,0.76),-0.31 (-1.25,0.63), -0.78 (-1.83,0.27), and -0.58 (-1.68,0.52), respectively) or CRVE (-0.31 (-2.20,1.57),-0.16 (-1.65,1.32),-0.79 (-2.45,0.87), and-0.47 (-2.21,1.26), respectively) (Figure 4.2).

Dietary AGEs and plasma biomarkers of endothelial dysfunction

With the individual biomarkers combined into a single z-score, greater intake of CEL, but not CML, MG-H1, or the combined dietary AGE z-score was associated with lower endothelial dysfunction plasma biomarker z-score in the fully adjusted model, with beta (SD) (95% CI) of -0.04 (-0.08,-0.00), 0.01 (-0.05,0.06), -0.02 (-0.07,0.02), and -0.03 (-0.08,0.02), respectively (Figure 4.2). Dietary CML, CEL, MG-H1, and the combined dietary AGE z-score were not associated with any of the individual plasma biomarkers (Supplementary table 4.9).

Dietary AGEs, the skin microvascular hyperemic response, and albuminuria

In the fully adjusted model, dietary AGEs CML, CEL, MG-H1, and the combined dietary AGE z-score were not associated with the heat-induced skin hyperemic response, with beta (%-change over baseline) (95% CI) of -36.67 (-96.01,22.66), -24.15 (-71.50,23.20), -20.26 (-71.08,30.57), and -29.94 (-84,03,24.16), respectively, and 24-hour albumin excretion, with beta (mg/24h, log-transformed) (95% CI) of -0.03 (-0.08,0.02), -0.03 (-0.07,0.01), -0.04 (-0.09,0.01), and -0.04 (-0.09,0.01), respectively (Figure 4.2).

Sensitivity analyses

In general, further adjustment of model 2 for either physical activity or markers of microangiopathy did not significantly alter the observed effect sizes of the aforementioned associations. However, with further adjustment for physical activity (analyses in which fewer participants were available), statistical significance for the associations of CEL intake with the endothelial function biomarker z-score (n = 2405) and flicker light-induced venular dilation (n = 1817) were already lost in the crude model (Supplementary Tables 4.10-13). All other sensitivity analyses did not materially change the results (data not shown).

Interaction analyses

In general, the observed associations between dietary AGEs and microvascular function were not modified by age, sex, BMI, glucose metabolism status, or eGFR (p for interaction > 0.05). Although, the association between dietary CML and MG-H1 and CRVE was significantly modified by presence of T2DM (p for interaction = 0.04), the association between dietary CML and MG-H1 and CRVE in this subgroup was not statistically significantly different compared to associations among people with NGM or prediabetes (Supplementary Table 4.14). Contrarily, greater MG-H1 intake was associated with lower CRVE (beta (µm) (95% CI) of -3.39 (-6.62,-0.15) in participants with prediabetes (Supplementary Table 4.14).

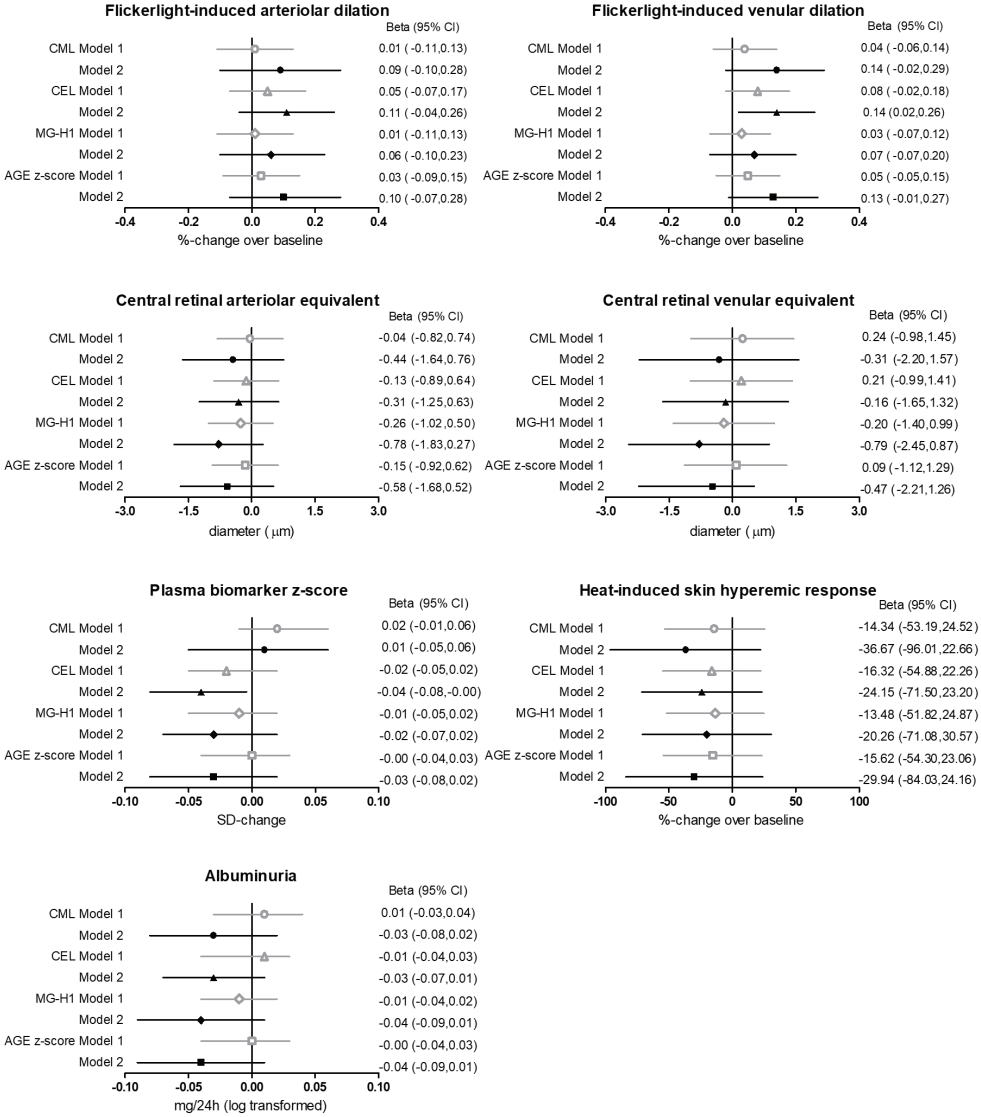

Figure 4.2 Multivariate-adjusted associations of dietary AGEs (in SD/day) and microvascular measurements tested with multiple linear regression analysis. Beta's and their (95% CIs) indicate the difference in microvascular outcome per 1 SD change in dietary AGE intake.

Model 1: Adjusted for participant characteristics: age, sex, glucose metabolism status.

Model 2: Additionally adjusted for cardiovascular risk factors and lifestyle factors – waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, office systolic blood pressure, and use of antihypertensive medication, caloric intake, educational level, alcohol intake, and the Dutch Healthy Diet index.

For albuminuria, model 2 is additionally adjusted for estimated glomerular filtration rate. Sample sizes (*n*): flicker light-induced arteriolar dilation: 2124, flicker light-induced venular dilation; 2158, central retinal arteriolar and venular equivalent: 2736, plasma biomarker z-score: 3110, heat-induced skin hyperemia: 1527, and albuminuria: 3123.

Abbreviations: CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Table 4.1 Characteristics of those with information on all confounders and at least one microvascular outcome, stratified by overall dietary AGE intake

Characteristics	Total sample (*n* = 3140)	Dietary AGE quartiles (z-score al all dietary AGEs)			
		Q1 (*n* = 786)	**Q2** (*n* = 786)	**Q3** (*n* = 786)	**Q4** (*n* = 786)
Demographics					
Age (years)	59.9 ± 8.2	60.7 ± 8.1	60.0 ± 8.4	59.6 ± 8.1	59.2 ± 8.1
Males (%)	51.0	35.7	48.7	52.8	66.5
Education level: Low/Medium/High (%)	33.5/28.2/38.3	38.0/26.1/35.9	32.3/27.9/39.9	34.0/28.6/37.4	29.9/30.2/39.9
Glucose metabolism status: NGM/prediabetes/T2DM/other (%)	56.6/14.7/ 27.7/1.0	55.3/13.6/ 30.1/1.0	55.9/13.9/ 29.5/0.8	58.0/15.8/ 25.1/1.1	57.1/15.5/ 26.1/1.3
Lifestyle					
Smoking: Never/Former/Current (%)	35.0/52.2/12.8	32.9/52.7/14.4	36.0/49.9/14.1	35.5/52.5/12.0	35.8/53.7/10.6
Waist circumference (cm)					
Males	101.5 ± 12.0	103.0 ± 11.8	101.6 ± 12.1	101.6 ± 11.5	100.6 ± 12.5
Females	89.8 ± 12.8	90.1 ± 13.5	88.8 ± 11.5	89.7 ± 12.5	91.2 ± 13.8
BMI (kg/m^2)	27.0 ± 4.5	27.1 ± 4.7	26.8 ± 4.3	27.0 ± 4.4	27.2 ± 4.7
Physical activity (min/day)[1]	119.3 ± 42.0	115.3 ± 43.4	116.5 ± 38.7	118.5 ± 39.6	127.0 ± 45.3
Biological					
Office Systolic BP (mmHg)	135.0 ± 18.2	134.7 ± 18.9	133.9 ± 18.2	135.5 ± 18.4	135.9 ± 17.4
Office Diastolic BP (mmHg)	76.1 ± 9.9	75.4 ± 9.9	75.3 ± 9.8	76.8 ± 9.8	77.0 ± 10.0
Anti-hypertensive medication (% yes)	40.3	44.3	40.2	38.9	37.9
Total/HDL-cholesterol ratio	3.7 ± 1.2	3.6 ± 1.2	3.6 ± 1.2	3.7 ± 1.1	3.8 ± 1.2
Triglycerides (mmol/L)	1.4 ± 0.9	1.4 ± 0.8	1.4 ± 0.9	1.4 ± 0.8	1.4 ± 0.9
Lipid-modifying medication (% yes)	36.5	38.6	38.0	34.5	35.0
Glucose-lowering medication (% yes)					
Insulin	6.8	8.0	6.9	6.5	6.0
Oral	20.2	21.2	21.8	18.8	19.1
eGFR (mL/min/1.73 m^2)[2]	88.0 ± 15.1	86.8 ± 15.4	88.3 ± 14.5	88.1 ± 14.6	88.6 ± 15.1
Retinopathy (% yes)[2]	1.6	1.0	2.0	1.5	2.1
Medical history of CVD (% yes)[2]	16.9	17.2	18.1	17.0	15.3
Dietary intake					
Energy intake (kcal/day)	2182 ± 606	1620 ± 377	1988 ± 348	2302 ± 400	2819 ± 523
Alcohol (g/day)	8.5 [1.4;18.8]	7.2 [0.5;18.6]	8.4 [1.5;17.6]	8.6 [1.7;17.3]	9.4 [2.5;20.5]
Coffee (g/day)	468.3 ± 296.9	418.9 ± 259.0	453.3 ± 274.9	476.8 ± 300.0	524.1 ± 338.5
Tea (g/day)	139.8 [13.0,325.0]	132.4 [7.5,325.0]	139.8 [17.5,355.0]	125.0 [19.4,325.0]	162.5 [19.4,346.1]
Dutch Healthy Diet Index[3]	76.0 ± 14.2	78.2 ± 14.2	77.0 ± 13.8	75.4 ± 14.2	73.4 ± 14.3
Dietary CML (mg/day)	3.3 ± 1.1	2.0 ± 0.4	2.8 ± 0.3	3.5 ± 0.4	4.7 ± 0.9
Dietary CEL (mg/day)	3.0 ± 1.2	1.8 ± 0.4	2.5 ± 0.3	3.1 ± 0.4	4.5 ± 1.4
Dietary MG-H1 (mg/day)	24.3 ± 8.8	15.5 ± 3.1	21.0 ± 2.5	25.7 ± 3.0	35.1 ± 8.8
Retinal microvascular measurements[4]					
Flickerlight-induced arteriolar dilation response (%)	3.0 ± 2.8	3.0 ± 2.8	2.9 ± 2.8	3.1 ± 2.8	3.2 ± 2.8
Flickerlight-induced venular dilation response (%)	3.9 ± 2.2	3.9 ± 2.3	3.7 ± 2.1	3.8 ± 2.2	4.0 ± 2.2
CRAE (µm)	142.3 ± 20.3	142.8 ± 20.8	142.9 ± 20.5	142.4 ± 19.7	141.2 ± 20.1
CRVE (µm)	214.6 ± 31.4	214.3 ± 32.8	214.2 ± 30.8	215.9 ± 30.5	213.7 ± 31.4
Plasma biomarkers of endothelial dysfunction[5]					
sICAM-1 (ng/ml)	354.2 ± 99.9	362.2 ± 112.9	352.9 ± 93.5	351.6 ± 90.4	350.2 ± 101.0
sVCAM-1 (ng/ml)	428.2 ± 101.3	429.8 ± 111.8	426.0 ± 101.4	426.2 ± 90.8	430.9 ± 101.3
E-Selectin (ng/ml)	118.3 ± 65.2	117.9 ± 66.7	117.0 ± 71.1	119.1 ± 59.9	118.3 ± 65.2
Von Willebrand Factor (%)	132.6 ± 48.4	134.3 ± 51.1	131.9 ± 47.1	132.0 ± 48.4	132.4 ± 47.0
Biomarker z-score (SD)	0.0 ± 1.0	0.0 ± 1.1	-0.0 ± 1.0	-0.0 ± 0.9	-0.0 ± 1.0
Skin microvascular measurements[6]					
Baseline skin blood flow (PU)	11.1 ± 6.5	11.3 ± 7.2	10.9 ± 6.1	11.1 ± 6.5	11.2 ± 6.0
Skin hyperemic response (%)	1124.4 ± 774.1	1155.8 ± 788.7	1140.6 ± 759.0	1129.8 ± 793.0	1069.9 ± 752.8
Kidney microvascular measurements					
Albuminuria (mg/24 h)[2]	6.7 [4.0;11.9]	6.9 [4.2;12.8]	6.5 [3.8;11.8]	6.4 [3.9;10.7]	7.1 [4.3;12.3]

Data are presented as means ± SD, medians [interquartile range], or percentages. [1]Physical activity data *n* = 2434. [2]Markers of microangiopathy *n* = 3048. [3]Modified version of the Dutch Healthy diet index that does not include alcohol intake. [4]Static retinal imaging *n* = 2736, dynamic retinal imaging *n* = 2124 for arteriolar dilation and *n* = 2158 for venular dilation. [5]Plasma biomarkers of endothelial dysfunction *n* = 3110. [6]Skin microvascular measurements *n* = 1527. Abbreviations: BP: blood pressure. CEL: N$^{\varepsilon}$-(1-carboxyethyl)lysine. CML: N$^{\varepsilon}$-(carboxymethyl)lysine. CRAE: central retinal arteriolar equivalent. CRVE: central retinal venular equivalent. eGFR: estimated glomerular filtration rate. MG-H1: N^{6}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. Other types of diabetes: Type 1 diabetes or surgery/medicine-induced. sICAM-1: intracellular adhesion molecule-1. sVCAM-1: vascular adhesion molecule-1. T2DM: type 2 diabetes mellitus.

Discussion

In this cross-sectional study, we are the first to investigate the association between three dietary AGEs, measured in selected food items using UPLC-MS/MS, with a broad array of microvascular measurements in a population-based cohort. We observed no consistent associations between habitual intake of dietary AGEs CML, CEL, and MG-H1 and several microvascular measurements. While there was a statistically significant association between higher intake of CEL, higher flicker light-induced venular dilation, and lower z-score of combined plasma endothelial dysfunction biomarkers, this did not apply to the individual plasma biomarkers and the effect sizes were small.

Increasing evidence suggests that AGEs of dietary origin contribute to the body AGE pool. It is thought that dietary AGEs, consumed as whole proteins, undergo digestion in the gastrointestinal tract and subsequently enter the circulation in their free form[7]. In the circulation, these free AGEs are in direct contact with the vascular endothelium and could potentially modulate microvascular function[9]. AGEs are involved in endothelial function, at least partly, through binding to the receptor for AGEs (RAGE) expressed by endothelial cells, which subsequently leads to generation of reactive oxygen species and activation of nuclear factor-κB[32]. However, this mechanism does not apply to free AGEs of dietary origin, as only protein-bound AGEs seem to have affinity for RAGE[33]. In line with this, it is still uncertain whether dietary AGEs have any (harmful) consequences. While meta-analyses suggest that a diet high in AGEs is linked to insulin resistance[34], increased inflammatory markers[35], and atherogenic dyslipidemia[8], they also conclude that high quality trials are lacking[8,35] and several individual studies found no such effects[10, 36-38]. Additionally, individual AGEs can potentially elicit different biological effects. While we present the combined dietary AGE z-score for easier interpretation of overall dietary AGE intake, it is important to consider that this may lead to loss of information. For example, biological effects of specific dietary AGEs could partly be mediated by interactions with the gut microbiome. While some strains of *E. coli* were shown to degrade CML into several metabolites with unknown biological effects[39], such effects have not yet been described for CEL and MG-H1.

We are the first to investigate associations between dietary AGEs and the retinal microcirculation in humans. We found no associations between dietary AGEs, central retinal arteriolar and venular diameters, and flicker light-induced arteriolar dilation, but surprisingly there was a small but statistically significant association between higher CEL intake and higher flicker light-induced venular dilation. Lower flicker light-induced retinal venular dilation was recently shown an independent

predictor of all-cause mortality in end-stage renal disease patients[40]. Factors that contribute to this response are inflammation, endothelial function, and NO bioavailability[41]. As previously mentioned, studies investigating the effect of dietary AGEs on inflammation have yielded conflicting results[10, 35-38]. We observed no robust associations between dietary AGEs and the central retinal venular diameter, which is also linked to systemic inflammation[42], or any of the other endothelium-dependent microvascular outcomes. Furthermore, statistical significance for the association between dietary CEL and flicker light-induced venular dilation was not consistent in sensitivity analyses with less participants. In light of the above, its biological relevance can be questioned.

We showed a statistically significant but small association of a higher intake of CEL, but not CML, MG-H1, or the combined dietary AGE z-score, with lower levels of the combined plasma endothelial dysfunction biomarkers z-score. We found no associations between dietary AGEs and the individual plasma biomarkers. These findings are partly in disagreement with previous studies, although results so far have been inconsistent. Whereas some authors reported a decrease in sVCAM-1 after a low AGE diet and an increase after a high AGE diet[43-45], others found no difference[10]. There are several possible explanations for the discrepancies between these studies and the present study. Next to differences in study design (cross sectional vs. a controlled dietary intervention), our analyses were performed in a larger sample, and we investigated three dietary AGEs as assessed by UPLC-MS/MS, as opposed to only CML assessed by immunohistochemistry. Additionally, we included not only sVCAM-1 but also sICAM-1, soluble eSelectin, and vWf. However, again, the observed effect size is small and thus its biological relevance may be questioned.

We found no association between intake of dietary AGEs and the heat-induced skin hyperemic response. This is in agreement with a previous study, where no associations were found between dietary AGEs, assessed by a three-day food record, and either post-occlusive or heat-induced skin hyperemia in 51 patients with chronic kidney disease and 51 controls[46]. In contrast, Negrean et al showed an acute impairment in post-occlusive skin hyperemia in 20 patients with T2DM after a fried/broiled high AGE meal compared to a steamed/boiled low AGE meal[47]. However, due to the extreme differences in preparation methods, the carcinogenic compound acrylamide or reduced micronutrient bioavailability may also contribute to the observed differences[48]. In line with this, the authors were not able to reproduce this impairment with administration of a specifically-designed low or high AGE drink[49].

Finally, we found no association between dietary AGEs and albuminuria. Although there is little research to compare to, our findings are in agreement with findings

by Harcourt et al whom observed no difference in albuminuria in 11 healthy but overweight individuals after a 2-week high AGE diet[50]. Albuminuria is an estimate not only of renal microvascular dysfunction[29], but also of several other microvascular pathologies[51, 52]. As such, it may act as a marker of generalized endothelial dysfunction[53], and the null finding in the present study strengthens the observed lack of associations between dietary AGEs and our other microvascular outcomes.

It is important to consider whether a potentially low consumption of dietary AGEs may influence the ability to detect an association. However, AGE intake in the current cohort is largely comparable to that of other Dutch cohorts in which associations between dietary AGEs and several outcomes have been reported[7, 54, 55]. Furthermore, the difference in intake between opposing quartiles of our cohort is approximately double that of the difference between the low and high AGE diet of a recent well-executed cross-over randomized controlled trial (RCT) in humans that observed a change in insulin sensitivity[56]. As such, dietary AGE intake in our cohort should be sufficient to detect an association with an outcome.

The current study has several strengths. Primarily, we investigated associations between dietary AGEs and measurements from several microvascular beds in a large study sample. Combined with the extensive phenotyping and population-based approach of The Maastricht Study, this enables us to draw conclusions on generalized microvascular function while controlling for several possible confounders. In addition, we used our UPLC-MS/MS based dietary AGE database[6] and a validated FFQ to capture the majority of sources of AGEs. The FFQ covers > 96% of total energy intake and a large list of nutrients[12], and has been updated with several high AGE products that are commonly consumed in our cohort (blood sausage, toasted bread, beef stew). Lastly, where biological effects of dietary AGEs are often investigated under extreme circumstances (heated and non-heated food products), our FFQ-based approach measures habitual intake.

The current study also has several limitations. Several uncertainties may negatively impact the accuracy of estimated dietary AGE intake. The use of FFQs may introduce recall bias[57] and our FFQ and AGE database do not include detailed information about food preparation for all foods. Although this only applied to a minority of food products within the AGE database, food preparation, referring mainly to cooking techniques and heating duration, largely determines the quantity of AGEs in food[58]. However, this will apply to all studies that use this approach, and our estimates of dietary AGE intake are in line with these other cohorts[54, 59, 60]. Nonetheless, combining our dietary AGE database and an FFQ is currently the most accurate method to determine dietary AGE intake in larger cohorts. Despite our cross-sectional analyses

being adjusted for a comprehensive set of a priori defined covariates, we cannot completely rule out residual confounding. Additionally, although we generally did not observe effect modification by age, sex, BMI, glucose metabolism status, or kidney function, our research question should be formally tested in other populations

In summary, we did not find any strong association between habitual intake of dietary AGEs and generalized microvascular function. Nonetheless, our findings would best require confirmation by longitudinal studies and RCTs in which participants receive specifically-designed dietary interventions in order to minimize residual confounding by certain food products or the preparation methods.

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Supplementary Methods

24-hour Ambulatory blood pressure measurement

Ambulatory blood pressure (BP) was measured with ambulatory 24-hour BP monitoring (WatchBP O3, Microlife AG, Switzerland). Cuffs were applied to the participants' non-dominant arm. Measurements were programmed for every 15 minutes during daytime (08.00–23.00 hours) and every 30 minutes during the night (23.00–08.00 hours), for a total of 24 hours. As quality criteria, mean 24-hour blood pressure measurements were only calculated if more than 14 valid measurements at daytime and more than 7 valid measurements at night were available, based on recommendations of the British Hypertension Society[1]. 24-hour ambulatory heart rate, and 24-hour ambulatory mean arterial pressure (MAP, defined as aDBP + (0.412 x aPP))[2] were calculated based on hourly averages[3].

Accelerometer data

Physical activity was measured using the activPAL3 physical activity monitor (PAL Technologies, Glasgow, UK). The activPAL3 is a small (53×35×7 mm), lightweight (15 g) triaxial accelerometer that records movement in the vertical, anteroposterior and mediolateral axes, and also determines posture (sitting or lying, standing and stepping) based on acceleration information. The device was attached directly to the skin on the front of the right thigh with transparent 3M Tegaderm tape, after the device had been waterproofed using a nitrile sleeve. Participants were asked to wear the accelerometer for 8 consecutive days, without removing it at any time. To avoid inaccurately identifying non-wear time, participants were asked not to replace the device once removed. Data were uploaded using the activPAL software and processed using customised software written in MATLAB R2013b (MathWorks, Natick, MA, USA). Data from the first day were excluded from the analysis because participants performed physical function tests at the research centre after the device was attached. In addition, data from the final wear day providing ≤14 waking hours of data were excluded from the analysis. Participants were included if they provided at least 1 valid day (>14 h of waking data).

References to Supplementary Methods

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Supplementary Tables

Supplementary Table 4.1 Mean intake of dietary AGEs (mg/day) in Dutch cohort studies

Cohort	**CML** Mean ± SD	**CEL** Mean ± SD	**MG-H1** Mean ± SD
The Maastricht Study	3.3 ± 1.1	3.0 ± 1.2	24.3 ± 8.8
CODAM[4]	3.1 ± 1.0	2.3 ± 0.8	21.7 ± 6.7
Rotterdam Study[5]	3.4 ± 0.9	3.1 ± 0.9	29.0 ± 7.9

Numbers indicate mean ± SD daily intake of dietary AGEs in other cohorts.

Supplementary Table 4.2 Population characteristics per measure of microvascular function

Characteristics	Retinal vessel average dilation	Retinal vessel calibers	Plasma biomarkers of endothelial dysfunction	Heat-induced skin hyperemic response	Albuminuria
Sample size (n)	2158	2736	3098	1527	3102
Demographics					
Age (years)	59.7 ± 8.2	59.9 ± 8.1	59.9 ± 8.2	60.2 ± 8.1	59.9 ± 8.2
Males (%)	50	51	51	52	51
Education level: Low/Medium/High (%)	33/28/39	33/29/38	33/28/39	33/28/39	33/28/39
Glucose metabolism status: NGM/prediabetes/T2DM/other[1] (%)	57/15/27/1	56/15/28/1	56/15/28/1	54/15/29/2	56/15/28/1
Lifestyle					
Smoking: Never/Former/Current (%)	36/52/12	35/53/12	35/52/13	33/55/12	35/52/13
Waist circumference (cm)					
Males	101.5 ± 12.0	101.5 ± 12.0	101.5 ± 12.0	101.4 ± 12.0	101.5 ± 12.0
Females	89.1 ± 12.5	89.7 ± 12.8	89.8 ± 12.9	90.2 ± 12.6	89.8 ± 12.8
BMI (kg/m^2)	26.9 ± 4.5	27.0 ± 4.5	27.1 ± 4.5	27.0 ± 4.5	27.0 ± 4.5
Physical activity (min/day)	120.4 ± 41.8	119.1 ± 41.9	119.4 ± 42.0	118.3 ± 41.8	119.3 ± 42.0
Biological					
Systolic BP (mmHg)	134.9 ± 18.1	134.9 ± 18.1	135.0 ± 18.2	135.8 ± 18.3	135.1 ± 18.3
Diastolic BP (mmHg)	76.2 ± 9.9	76.1 ± 9.9	76.1 ± 9.9	76.5 ± 9.6	76.1 ± 9.9
Anti-hypertensives (%)	39	41	41	42	40
Total/HDL-cholesterol ratio	3.6 ± 1.1	3.6 ± 1.1	3.7 ± 1.2	3.7 ± 1.1	3.7 ± 1.2
Triglycerides (mmol/L)	1.4 ± 0.8	1.4 ± 0.8	1.4 ± 0.9	1.4 ± 0.9	1.4 ± 0.9
Lipid-modifying medication (%-yes)	36	37	37	39	37
Glucose-lowering medication (%-yes)					
Insulin	6	7	7	7	7
Oral	20	21	20	22	20
eGFR (mL/min/1.73 m^2)	88.2 ± 14.6	88.2 ± 14.8	88.0 ± 14.9	88.2 ± 14.6	88.0 ± 14.9
Retinopathy (% yes)	2	2	2	2	2
Medical history of CVD (%)	16	17	17	18	17
Dietary intake					
Energy intake (kcal/day)	2168 ± 596	2181 ± 607	2182 ± 603	2180 ± 590	2183 ± 604
Alcohol (g/day)	8.5 [1.6-18.8]	8.6 [1.5-18.9]	8.5 [1.5-18.8]	8.8 [1.4-19.2]	8.5 [1.5-18.9]
Coffee (g/day)	463.1 ± 291.4	469.4 ± 296.6	468.5 ± 297.6	468.1 ± 292.4	468.8 ± 296.6
Tea (g/day)	142.5 [17.5,355.0]	142.5 [13.0,325.0]	139.8 [13.0,325.0]	139.8 [10.0,325.0]	139.8 [13.0,325.0]
Dutch Healthy Diet Index[2]	76.6 ± 14.3	76.4 ± 14.2	76.0 ± 14.2	76.0 ± 14.1	76.0 ± 14.2
Dietary CML (mg/day)	3.3 ± 1.2	3.3 ± 1.2	3.3 ± 1.1	3.3 ± 1.1	3.3 ± 1.1
Dietary CEL (mg/day)	3.0 ± 1.2	3.0 ± 1.3	3.0 ± 1.2	3.0 ± 1.2	3.0 ± 1.2
Dietary MG-H1 (mg/day)	24.3 ± 8.9	24.4 ± 8.9	24.3 ± 8.8	24.3 ± 8.8	24.3 ± 8.8
Retinal microvascular measurements					
Flickerlight-induced arteriolar dilation response (%)	3.0 ± 2.8	3.1 ± 2.8	3.0 ± 2.8	2.9 ± 2.8	3.0 ± 2.8
Flickerlight-induced venular dilation response (%)	3.9 ± 2.2	3.9 ± 2.2	3.9 ± 2.2	3.8 ± 2.1	3.9 ± 2.2
CRAE (µm)	142.9 ± 19.7	142.3 ± 20.3	142.3 ± 20.3	143.2 ± 20.0	142.3 ± 20.3
CRVE (µm)	215.1 ± 31.2	214.5 ± 31.4	214.6 ± 31.3	215.2 ± 32.0	214.5 ± 31.3
Plasma Biomarkers of endothelial dysfunction					
sICAM-1 (ng/ml)	356.7 ± 99.9	355.6 ± 99.2	354.2 ± 99.9	357.5 ± 96.2	354.1 ± 99.6
sVCAM-1 (ng/ml)	429.7 ± 102.5	429.0 ± 100.5	428.4 ± 101.5	435.4 ± 101.1	428.1 ± 101.3
E-Selectin (ng/ml)	117.8 ± 67.1	118.6 ± 66.3	118.0 ± 65.9	117.9 ± 67.6	118.0 ± 65.6
Von Willebrand Factor (%)	132.4 ±48.8	131.9 ± 48.1	132.5 ± 48.4	132.5 ± 47.3	132.3 ± 48.2
Skin microvascular measurements					
Baseline skin blood flow (PU)	11.2 ± 6.6	11.2 ± 6.5	11.1 ± 6.5	11.1 ± 6.5	11.1 ± 6.5
Skin hyperemic response (%)	1125 ± 781	1116 ± 782	1122 ± 772	1124 ± 774	1122 ± 773
Kidney microvascular measurements					
Albuminuria (mg/24 h)	6.5 [3.9-11.7]	6.6 [3.9-12.0]	6.7 [4.0-12.0]	7.0 [4.2-11.8]	6.7 ± 4.0-12.0]

Data are presented as means ± SD, medians [interquartile range], or percentages. [1] Other types of diabetes: Type 1 diabetes or surgery/medicine induced. [2] Modified version of the Dutch Healthy Diet Index without alcohol. Abbreviations: NGM: normal glucose metabolism. T2DM: type 2 diabetes mellitus. eGFR: estimated glomerular filtration rate. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. CML: N^{ε}-(carboxymethyl)lysine. sICAM-1: intracellular adhesion molecule-1. sVCAM-1: vascular adhesion molecule-1. CRAE: central retinal arteriolar equivalent. CRVE: central retinal venular equivalent.

Supplementary Table 4.3 Characteristics of those included in the analysis of retinal arteriolar average dilation compared to those excluded from the analysis

Characteristics	Included (*n* = 2124)	Excluded (*n* = 1327)	*P*
Demographics			
Age (years)	59.7 ± 8.2	59.9 ± 8.4	0.41
Males (%)	50.1	53.5	0.06
Education level: Low/Medium/High (%)	32.1/28.8/39.1	36.2/27.3/36.5	0.05
Glucose metabolism status: NGM/preD/T2DM/other (%)	57.1/14.7/27.2/1.1	53.7/15.0/30.0/1.4	0.21
Lifestyle			
Smoking: Never/Former/Current (%)	35.6/52.4/12.0	32.5/50.6/16.9	<0.01
Waist circumference (cm)			
Males	101.3 ± 12.0	101.8 ± 12.2	0.42
Females	89.2 ± 12.5	91.5 ± 13.7	<0.01
BMI (kg/m^2)	26.9 ± 4.5	27.4 ± 4.7	<0.01
Physical activity (min/day)	120.7 ± 41.8	115.6 ± 42.8	<0.01
Biological			
Office Systolic BP (mmHg)	134.8 ± 18.1	135.4 ± 18.3	0.39
Office Diastolic BP (mmHg)	76.2 ± 9.9	76.1 ± 9.8	0.85
Anti-hypertensive medication (% yes)	38.6	43.1	0.01
Total/HDL-cholesterol ratio	3.6 ± 1.1	3.8 ± 1.2	<0.01
Triglycerides (mmol/L)	1.4 ± 0.8	1.4 ± 0.9	0.63
Lipid-modifying medication (% yes)	35.7	37.7	0.26
Glucose-lowering medication (% yes)			
Insulin	6.3	9.3	<0.01
Oral	20.1	21.9	0.20
eGFR (mL/min/1.73 m^2)	88.3 ± 14.6	87.9 ± 15.4	0.50
Retinopathy (% yes)	1.9	2.1	0.79
Medical history of CVD (% yes)	15.8	18.4	0.06
Dietary intake			
Energy intake (kcal/day)	2168 ± 592	2207 ± 633	0.08
Alcohol (g/day)	8.6 [1.6-19.0]	7.6 [1.1-17.8]	0.16
Coffee (g/day)	463.0 ± 293.7	474.6 ± 303.7	0.30
Tea (g/day)	142.5 [17.5,346.1]	125.0 [10.0,325.0]	0.02
Dutch Healthy Diet Index [1]	76.6 ± 14.3	74.7 ± 14.0	<0.01
Dietary CML (mg/day)	3.3 ± 1.1	3.3 ± 1.1	0.84
Dietary CEL (mg/day)	3.0 ± 1.2	3.0 ± 1.3	0.78
Dietary MG-H1 (mg/day)	24.3 ± 8.8	24.2 ± 8.6	0.73
Retinal microvascular measurements			
Flickerlight-induced arteriolar dilation response (%)	3.0 ± 2.8	2.5 ± 2.8	<0.01
Flickerlight-induced venular dilation response (%)	3.9 ± 2.2	3.6 ± 2.0	0.03
CRAE (μm)	142.7 ± 19.7	141.0 ± 22.1	0.04
CRVE (μm)	215.0 ± 30.9	213.5 ± 32.5	0.25
Plasma biomarkers of endothelial dysfunction			
sICAM-1 (ng/ml)	356.9 ± 100.3	351.9 ± 98.9	0.15
sVCAM-1 (ng/ml)	429.7 ± 103.0	426.7 ± 99.5	0.40
E-Selectin (ng/ml)	117.8 ± 67.2	119.7 ± 61.7	0.41
Von Willebrand Factor (%)	132.5 ± 49.0	133.6 ± 47.7	0.52
Skin microvascular measurements			
Baseline skin blood flow (PU)	11.1 ± 6.3	11.1 ± 6.7	0.77
Skin hyperemic response (%)	1130.5 ± 781.0	1100.7 ± 744.6	0.54
Kidney microvascular measurements			
Albuminuria (mg/24 h)	6.4 [3.9-11.6]	7.2 [4.5-12.8]	<0.01

Data are presented as means ± SD, medians [interquartile range], or percentages. Participants that were excluded from the analyses due to missing covariates were compared with the included participants by means of an ANOVA or chi-squared test, as appropriate. [1] Modified version of the Dutch Healthy Diet Index that does not include alcohol intake. Abbreviations: CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. CRAE: central retinal arteriolar equivalent. CRVE: central retinal venular equivalent. eGFR: estimated glomerular filtration rate. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. Other types of diabetes: Type 1 diabetes or surgery/medicine-induced. sICAM-1: intracellular adhesion molecule-1. sVCAM-1: vascular adhesion molecule-1. T2DM: type 2 diabetes mellitus.

Supplementary Table 4.4 Characteristics of those included in the analysis of retinal venular average dilation compared to those excluded from the analysis

Characteristics	Included (*n* = 2158)	Excluded (*n* = 1293)	*P*
Demographics			
Age (years)	59.7 ± 8.2	59.8 ± 8.4	0.74
Males (%)	50.0	53.9	0.03
Education level: Low/Medium/High (%)	32.8/28.5/38.7	35.0/27.9/37.1	0.41
Glucose metabolism status: NGM/preD/T2DM/other (%)	57.1/14.7/27.2/1.1	53.5/14.9/30.2/1.4	0.16
Lifestyle			
Smoking: Never/Former/Current (%)	35.8/52.3/11.9	32.2/50.7/17.1	<0.01
Waist circumference (cm)			
Males	101.5 ± 12.0	101.6 ± 12.3	0.86
Females	89.1 ± 12.5	91.6 ± 13.8	<0.01
BMI (kg/m^2)	26.9 ± 4.5	27.4 ± 4.7	<0.01
Physical activity (min/day)	120.4 ± 41.8	116.0 ± 43.0	0.01
Biological			
Office Systolic BP (mmHg)	134.9 ± 18.1	135.3 ± 18.4	0.57
Office Diastolic BP (mmHg)	76.2 ± 9.9	76.0 ± 9.7	0.62
Anti-hypertensive medication (% yes)	38.9	42.7	0.03
Total/HDL-cholesterol ratio	3.6 ± 1.1	3.8 ± 1.2	<0.01
Triglycerides (mmol/L)	1.4 ± 0.8	1.4 ± 0.9	0.90
Lipid-modifying medication (% yes)	36.0	37.3	0.47
Glucose-lowering medication (% yes)			
Insulin	6.3	9.4	0.01
Oral	20.2	21.8	0.26
eGFR (mL/min/1.73 m^2)	88.2 ± 14.6	88.0 ± 15.5	0.77
Retinopathy (% yes)	1.9	2.2	0.77
Medical history of CVD (% yes)	15.8	18.5	0.05
Dietary intake			
Energy intake (kcal/day)	2168 ± 595	2208 ± 628	0.08
Alcohol (g/day)	8.5 [1.6-18.8]	7.7 [1.1-18.1]	0.34
Coffee (g/day)	463.1 ± 291.4	474.8 ± 308.6	0.29
Tea (g/day)	142.5 [17.5,355.0]	125.0 [10.0,325.0]	0.01
Dutch Healthy Diet Index [1]	76.6 ± 14.3	74.6 ± 14.0	<0.01
Dietary CML (mg/day)	3.3 ± 1.1	3.3 ± 1.1	0.84
Dietary CEL (mg/day)	3.0 ± 1.2	3.0 ± 1.3	0.78
Dietary MG-H1 (mg/day)	24.3 ± 8.9	24.2 ± 8.6	0.69
Retinal microvascular measurements			
Flickerlight-induced arteriolar dilation response (%)	3.0 ± 2.8	2.5 ± 2.8	0.02
Flickerlight-induced venular dilation response (%)	3.9 ± 2.2	3.5 ± 1.9	0.04
CRAE (µm)	142.9 ± 19.7	140.6 ± 22.1	0.01
CRVE (µm)	215.1 ± 31.2	213.0 ± 31.9	0.10
Plasma biomarkers of endothelial dysfunction			
sICAM-1 (ng/ml)	356.7 ± 100.0	352.0 ± 99.0	0.18
sVCAM-1 (ng/ml)	429.7 ± 102.5	426.6 ± 100.3	0.40
E-Selectin (ng/ml)	117.8 ± 67.1	119.8 ± 61.8	0.38
Von Willebrand Factor (%)	132.4 ± 48.8	133.8 ± 48.1	0.39
Skin microvascular measurements			
Baseline skin blood flow (PU)	11.2 ± 6.6	10.9 ± 6.1	0.44
Skin hyperemic response (%)	1125.0 ± 780.8	1111.3 ± 743.8	0.74
Kidney microvascular measurements			
Albuminuria (mg/24 h)	6.5 [3.9-11.7]	7.2 [4.6-12.6]	<0.01

Data are presented as means ± SD, medians [interquartile range], or percentages. Participants that were excluded from the analyses due to missing covariates were compared with the included participants by means of an ANOVA or chi-squared test, as appropriate. [1] Modified version of the Dutch Healthy Diet Index that does not include alcohol intake. Abbreviations: CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. CRAE: central retinal arteriolar equivalent. CRVE: central retinal venular equivalent. eGFR: estimated glomerular filtration rate. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. Other types of diabetes: Type 1 diabetes or surgery/medicine-induced. sICAM-1: intracellular adhesion molecule-1. sVCAM-1: vascular adhesion molecule-1. T2DM: type 2 diabetes mellitus.

Supplementary Table 4.5 Characteristics of those included in the analysis of central retinal arteriolar and venular equivalent compared to those excluded from the analysis

Characteristics	Included (*n* = 2736)	Excluded (*n* = 715)	*P*
Demographics			
Age (years)	59.9 ± 8.1	59.4 ± 8.7	0.23
Males (%)	50.8	54.0	0.13
Education level: Low/Medium/High (%)	33.4/28.5/33.4	34.3/27.1/38.5	0.78
Glucose metabolism status: NGM/preD/T2DM/other (%)	56.3/14.8/27.9/1.0	53.6/15.0/29.5/2.0	0.15
Lifestyle			
Smoking: Never/Former/Current (%)	35.2/52.5/12.3	31.3/48.6/20.2	<0.01
Waist circumference (cm)			
Males	101.5 ± 12.0	101.6 ± 12.5	0.91
Females	89.7 ± 12.8	91.2 ± 13.7	0.08
BMI (kg/m^2)	27.0 ± 4.5	27.4 ± 4.8	0.05
Physical activity (min/day)	119.1 ± 41.9	119.2 ± 43.4	0.94
Biological			
Office Systolic BP (mmHg)	134.9 ± 18.1	135.7 ± 18.6	0.29
Office Diastolic BP (mmHg)	76.1 ± 9.9	76.2 ± 9.8	0.76
Anti-hypertensive medication (% yes)	40.6	39.2	0.49
Total/HDL-cholesterol ratio	3.6 ± 1.1	3.9 ± 1.3	<0.01
Triglycerides (mmol/L)	1.4 ± 0.8	1.4 ± 0.9	0.46
Lipid-modifying medication (% yes)	36.7	35.5	0.57
Glucose-lowering medication (% yes)			
Insulin	6.7	10.3	<0.01
Oral	20.6	21.4	0.64
eGFR (mL/min/1.73 m^2)	88.1 ± 14.8	87.9 ± 15.5	0.71
Retinopathy (% yes)	1.9	2.2	0.83
Medical history of CVD (% yes)	16.9	16.4	0.82
Dietary intake			
Energy intake (kcal/day)	2181 ± 607	2185 ± 605	0.89
Alcohol (g/day)	8.6 [1.5-18.9]	6.7 [0.9-16.6]	0.01
Coffee (g/day)	469.4 ± 296.6	453.2 ± 300.2	0.27
Tea (g/day)	142.5 [13.0,325.0]	107.5 [10.0,325.0]	0.02
Dutch Healthy Diet Index [1]	76.4 ± 14.2	73.7 ± 14.1	<0.01
Dietary CML (mg/day)	3.3 ± 1.2	3.2 ± 1.1	0.20
Dietary CEL (mg/day)	3.0 ± 1.3	2.8 ± 1.1	0.01
Dietary MG-H1 (mg/day)	24.4 ± 8.9	23.5 ± 7.9	0.03
Retinal microvascular measurements			
Flickerlight-induced arteriolar dilation response (%)	3.1 ± 2.8	2.4 ± 2.7	<0.01
Flickerlight-induced venular dilation response (%)	3.9 ± 2.2	3.5 ± 1.9	0.01
CRAE (μm)	142.3 ± 20.3	140.4 ± 22.6	0.18
CRVE (μm)	214.5 ± 31.4	214.3 ± 31.8	0.90
Plasma biomarkers of endothelial dysfunction			
sICAM-1 (ng/ml)	355.6 ± 99.2	352.6 ± 101.9	0.49
sVCAM-1 (ng/ml)	429.0 ± 100.5	426.6 ± 106.3	0.58
E-Selectin (ng/ml)	118.6 ± 66.3	118.3 ± 60.7	0.91
Von Willebrand Factor (%)	131.9 ± 48.1	136.7 ± 49.7	0.02
Skin microvascular measurements			
Baseline skin blood flow (PU)	11.2 ± 6.5	10.5 ± 5.8	0.08
Skin hyperemic response (%)	1116.1 ± 781.5	1140.6 ± 712.2	0.61
Kidney microvascular measurements			
Albuminuria (mg/24 h)	6.6 [3.9-12.0]	7.2 [4.6-12.2]	0.02

Data are presented as means ± SD, medians [interquartile range], or percentages. Participants that were excluded from the analyses due to missing covariates were compared with the included participants by means of an ANOVA or chi-squared test, as appropriate. [1] Modified version of the Dutch Healthy Diet Index that does not include alcohol intake. Abbreviations: CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. CRAE: central retinal arteriolar equivalent. CRVE: central retinal venular equivalent. eGFR: estimated glomerular filtration rate. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. Other types of diabetes: Type 1 diabetes or surgery/medicine-induced. sICAM-1: intracellular adhesion molecule-1. sVCAM-1: vascular adhesion molecule-1. T2DM: type 2 diabetes mellitus.

Supplementary Table 4.6 Characteristics of those included in the analysis of plasma biomarkers of endothelial dysfunction compared to those excluded from the analysis

Characteristics	Included (*n* = 3097)	Excluded (*n* = 354)	*P*
Demographics			
Age (years)	59.9 ± 8.2	58.6 ± 9.1	0.01
Males (%)	51.2	53.7	0.40
Education level: Low/Medium/High (%)	33.3/28.1/38.6	36.8/29.6/33.6	0.25
Glucose metabolism status: NGM/preD/T2DM/other (%)	56.6/14.7/27.7/1.0	48.6/15.5/33.3/1.0	0.01
Lifestyle			
Smoking: Never/Former/Current (%)	35.0/52.2/12.8	28.9/46.4/24.7	<0.01
Waist circumference (cm)			
Males	101.5 ± 12.0	101.3 ± 12.6	0.78
Females	89.8 ± 12.9	92.2 ± 14.2	0.03
BMI (kg/m^2)	27.1 ± 4.5	27.4 ± 4.9	0.18
Physical activity (min/day)	119.4 ± 42.0	115.8 ± 43.9	0.20
Biological			
Office Systolic BP (mmHg)	135.0 ± 18.2	135.8 ± 18.2	0.45
Office Diastolic BP (mmHg)	76.1 ± 9.9	76.2 ± 9.1	0.88
Anti-hypertensive medication (% yes)	40.5	38.7	0.53
Total/HDL-cholesterol ratio	3.7 ± 1.2	3.8 ± 1.2	0.02
Triglycerides (mmol/L)	1.4 ± 0.9	1.5 ± 0.9	0.53
Lipid-modifying medication (% yes)	36.6	35.0	0.56
Glucose-lowering medication (% yes)			
Insulin	6.9	12.5	<0.01
Oral	20.2	25.5	0.03
eGFR (mL/min/1.73 m^2)	88.0 ± 14.9	89.3 ± 15.3	0.15
Retinopathy (% yes)	2.0	2.0	0.83
Medical history of CVD (% yes)	16.9	16.0	0.75
Dietary intake			
Energy intake (kcal/day)	2181 ± 603	2180 ± 683	0.97
Alcohol (g/day)	8.5 [1.5-18.9]	4.6 [0.6-14.0]	<0.01
Coffee (g/day)	468.5 ± 297.6	428.8 ± 286.1	0.14
Tea (g/day)	139.8 [13.0,325.0]	139.8 [10.0,375.0]	0.84
Dutch Healthy Diet Index [1]	76.0 ± 14.2	75.2 ± 13.8	0.54
Dietary CML (mg/day)	3.3 ± 1.1	3.2 ± 1.1	0.32
Dietary CEL (mg/day)	3.0 ± 1.2	2.9 ± 1.3	0.51
Dietary MG-H1 (mg/day)	24.3 ± 8.8	24.1 ± 9.2	0.84
Retinal microvascular measurements			
Flickerlight-induced arteriolar dilation response (%)	3.0 ± 2.8	2.6 ± 2.8	0.03
Flickerlight-induced venular dilation response (%)	3.9 ± 2.2	3.5 ± 1.9	0.03
CRAE (μm)	142.3 ± 20.3	140.6 ± 22.1	0.18
CRVE (μm)	214.6 ± 31.3	214.0 ± 32.1	0.78
Plasma biomarkers of endothelial dysfunction			
sICAM-1 (ng/ml)	354.1 ± 99.9	363.2 ± 98.1	0.12
sVCAM-1 (ng/ml)	428.4 ± 101.6	430.0 ± 103.1	0.79
E-Selectin (ng/ml)	118.0 ± 65.9	123.7 ± 56.8	0.14
Von Willebrand Factor (%)	132.5 ± 48.4	136.7 ± 49.6	0.15
Skin microvascular measurements			
Baseline skin blood flow (PU)	11.1 ± 6.4	10.7 ± 5.7	0.38
Skin hyperemic response (%)	1122.2 ± 771.8	1106.3 ± 744.2	0.80
Kidney microvascular measurements			
Albuminuria (mg/24 h)	6.7 [4.0-12.0]	7.1 [4.2-12.3]	0.27

Data are presented as means ± SD, medians [interquartile range], or percentages. Participants that were excluded from the analyses due to missing covariates were compared with the included participants by means of an ANOVA or chi-squared test, as appropriate. [1] Modified version of the Dutch Healthy Diet Index that does not include alcohol intake. Abbreviations: CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. CRAE: central retinal arteriolar equivalent. CRVE: central retinal venular equivalent. eGFR: estimated glomerular filtration rate. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. Other types of diabetes: Type 1 diabetes or surgery/medicine-induced. sICAM-1: intracellular adhesion molecule-1. sVCAM-1: vascular adhesion molecule-1. T2DM: type 2 diabetes mellitus.

Supplementary Table 4.7 Characteristics of those included in the analysis of skin microvascular function compared to those excluded from the analysis

Characteristics	Included (*n* = 1527)	Excluded (*n* = 1924)	*P*
Demographics			
Age (years)	59.9 ± 8.2	58.6 ± 9.1	0.01
Males (%)	51.2	53.7	0.40
Education level: Low/Medium/High (%)	33.3/28.1/38.6	36.8/29.6/33.6	0.25
Glucose metabolism status: NGM/preD/T2DM/other (%)	56.6/14.7/27.7/1.0	48.6/15.5/33.3/1.0	0.01
Lifestyle			
Smoking: Never/Former/Current (%)	35.0/52.2/12.8	28.9/46.4/24.7	<0.01
Waist circumference (cm)			
Males	101.5 ± 12.0	101.3 ± 12.6	0.78
Females	89.8 ± 12.9	92.2 ± 14.2	0.03
BMI (kg/m^2)	27.1 ± 4.5	27.4 ± 4.9	0.18
Physical activity (min/day)	119.4 ± 42.0	115.8 ± 43.9	0.20
Biological			
Office Systolic BP (mmHg)	135.0 ± 18.2	135.8 ± 18.2	0.45
Office Diastolic BP (mmHg)	76.1 ± 9.9	76.2 ± 9.1	0.88
Anti-hypertensive medication (% yes)	40.5	38.7	0.53
Total/HDL-cholesterol ratio	3.7 ± 1.2	3.8 ± 1.2	0.02
Triglycerides (mmol/L)	1.4 ± 0.9	1.5 ± 0.9	0.53
Lipid-modifying medication (% yes)	36.6	35.0	0.56
Glucose-lowering medication (% yes)			
Insulin	6.9	12.5	<0.01
Oral	20.2	25.5	0.03
eGFR (mL/min/1.73 m^2)	88.0 ± 14.9	89.3 ± 15.3	0.15
Retinopathy (% yes)	2.0	2.0	0.83
Medical history of CVD (% yes)	16.9	16.0	0.75
Dietary intake			
Energy intake (kcal/day)	2181 ± 603	2180 ± 683	0.97
Alcohol (g/day)	8.5 [1.5-18.9]	4.6 [0.6-14.0]	<0.01
Coffee (g/day)	468.5 ± 297.6	428.8 ± 286.1	0.14
Tea (g/day)	139.8 [13.0,325.0]	139.8 [10.0,375.0]	0.84
Dutch Healthy Diet Index [1]	76.0 ± 14.2	75.2 ± 13.8	0.54
Dietary CML (mg/day)	3.3 ± 1.1	3.2 ± 1.1	0.32
Dietary CEL (mg/day)	3.0 ± 1.2	2.9 ± 1.3	0.51
Dietary MG-H1 (mg/day)	24.3 ± 8.8	24.1 ± 9.2	0.84
Retinal microvascular measurements			
Flickerlight-induced arteriolar dilation response (%)	3.0 ± 2.8	2.6 ± 2.8	0.03
Flickerlight-induced venular dilation response (%)	3.9 ± 2.2	3.5 ± 1.9	0.03
CRAE (µm)	142.3 ± 20.3	140.6 ± 22.1	0.18
CRVE (µm)	214.6 ± 31.3	214.0 ± 32.1	0.78
Plasma biomarkers of endothelial dysfunction			
sICAM-1 (ng/ml)	354.1 ± 99.9	363.2 ± 98.1	0.12
sVCAM-1 (ng/ml)	428.4 ± 101.6	430.0 ± 103.1	0.79
E-Selectin (ng/ml)	118.0 ± 65.9	123.7 ± 56.8	0.14
Von Willebrand Factor (%)	132.5 ± 48.4	136.7 ± 49.6	0.15
Skin microvascular measurements			
Baseline skin blood flow (PU)	11.1 ± 6.4	10.7 ± 5.7	0.38
Skin hyperemic response (%)	1122.2 ± 771.8	1106.3 ± 744.2	0.80
Kidney microvascular measurements			
Albuminuria (mg/24 h)	6.7 [4.0-12.0]	7.1 [4.2-12.3]	0.27

Data are presented as means ± SD, medians [interquartile range], or percentages. Participants that were excluded from the analyses due to missing covariates were compared with the included participants by means of an ANOVA or chi-squared test, as appropriate. [1] Modified version of the Dutch Healthy Diet Index that does not include alcohol intake. Abbreviations: CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. CRAE: central retinal arteriolar equivalent. CRVE: central retinal venular equivalent. eGFR: estimated glomerular filtration rate. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. Other types of diabetes: Type 1 diabetes or surgery/medicine-induced. sICAM-1: intracellular adhesion molecule-1. sVCAM-1: vascular adhesion molecule-1. T2DM: type 2 diabetes mellitus.

Supplementary Table 4.8 Characteristics of those included in the analysis of albuminuria compared to those excluded from the analysis

Characteristics	Included (*n* = 3102)	Excluded (*n* = 350)	*P*
Demographics			
Age (years)	60.0 ± 8.2	58.1 ± 8.9	<0.01
Males (%)	51.1	54.6	0.24
Education level: Low/Medium/High (%)	33.4/28.2/38.4	35.1/29.3/35.5	0.56
Glucose metabolism status: NGM/preD/T2DM/other (%)	56.5/14.8/27.6/1.1	49.1/14.9/33.7/2.3	0.02
Lifestyle			
Smoking: Never/Former/Current (%)	35.1/52.1/12.7	27.7/47.3/25.0	<0.01
Waist circumference (cm)			
Males	101.5 ± 12.0	101.6 ± 13.2	0.92
Females	89.8 ± 12.8	92.3 ± 14.7	0.02
BMI (kg/m^2)	27.0 ± 4.5	27.6 ± 5.1	0.04
Physical activity (min/day)	119.3 ± 42.0	116.8 ± 43.7	0.38
Biological			
Office Systolic BP (mmHg)	135.1 ± 18.3	135.0 ± 17.8	0.98
Office Diastolic BP (mmHg)	76.1 ± 9.9	76.4 ± 9.3	0.66
Anti-hypertensive medication (% yes)	40.4	39.4	0.73
Total/HDL-cholesterol ratio	3.7 ± 1.2	3.8 ± 1.2	0.02
Triglycerides (mmol/L)	1.4 ± 0.9	1.4 ± 0.9	0.87
Lipid-modifying medication (% yes)	34.3	36.7	0.38
Glucose-lowering medication (% yes)			
Insulin	6.8	12.9	<0.01
Oral	20.1	26.4	0.01
eGFR (mL/min/1.73 m^2)	88.0 ± 14.9	89.6 ± 15.3	0.06
Retinopathy (% yes)	1.9	2.4	0.51
Medical history of CVD (% yes)	16.8	16.2	0.87
Dietary intake			
Energy intake (kcal/day)	2182 ± 603	2156 ± 681	0.63
Alcohol (g/day)	8.5 [1.5-18.9]	4.2 [0.4-12.0]	<0.01
Coffee (g/day)	468.8 ± 296.6	420.8 ± 308.4	0.08
Tea (g/day)	139.8 [13.0,325.0]	142.5 [10.0,461.5]	0.60
Dutch Healthy Diet Index [1]	76.0± 14.2	75.3 ± 15.2	0.57
Dietary CML (mg/day)	3.3 ± 1.1	3.1 ± 1.1	0.20
Dietary CEL (mg/day)	3.0 ± 1.2	2.9 ± 1.3	0.32
Dietary MG-H1 (mg/day)	24.3 ± 8.8	23.5 ± 8.8	0.29
Retinal microvascular measurements			
Flickerlight-induced arteriolar dilation response (%)	3.0 ± 2.8	2.5 ± 2.8	0.01
Flickerlight-induced venular dilation response (%)	3.9 ± 2.2	3.6 ± 2.1	0.05
CRAE (μm)	142.3 ± 20.3	140.7 ± 22.1	0.21
CRVE (μm)	214.5 ± 31.3	214.5 ± 32.8	0.99
Plasma biomarkers of endothelial dysfunction			
sICAM-1 (ng/ml)	354.1 ± 99.6	363.2 ± 101.0	0.12
sVCAM-1 (ng/ml)	428.0 ± 101.3	433.3 ± 105.1	0.38
E-Selectin (ng/ml)	118.1 ± 65.6	123.0 ± 60.3	0.19
Von Willebrand Factor (%)	132.3 ± 48.2	138.5 ± 50.5	0.03
Skin microvascular measurements			
Baseline skin blood flow (PU)	11.1 ± 6.5	10.9 ± 5.8	0.72
Skin hyperemic response (%)	1121.8 ± 773.2	1109.4 ± 731.9	0.84
Kidney microvascular measurements			
Albuminuria (mg/24 h)	6.7 [4.0-12.0]	7.2 [4.3-12.5]	0.19

Data are presented as means ± SD, medians [interquartile range], or percentages. Participants that were excluded from the analyses due to missing covariates were compared with the included participants by means of an ANOVA or chi-squared test, as appropriate. [1] Modified version of the Dutch Healthy Diet Index that does not include alcohol intake. Abbreviations: CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. CRAE: central retinal arteriolar equivalent. CRVE: central retinal venular equivalent. eGFR: estimated glomerular filtration rate. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. Other types of diabetes: Type 1 diabetes or surgery/medicine-induced. sICAM-1: intracellular adhesion molecule-1. sVCAM-1: vascular adhesion molecule-1. T2DM: type 2 diabetes mellitus.

Supplementary Table 4.9 Associations between dietary AGEs (Beta (95% CI)) and plasma biomarkers of endothelial dysfunction.

Dietary AGE (SD/day)	**sICAM-1** (ng/L)	**sVCAM-1** (ng/L)	**eSelectin** (ng/L)	**vWf** (%)
CML				
Model 1	0.34 (-3.21,3.89)	2.16 (-1.44,5.76)	1.20 (-1.08,3.49)	0.97 (-0.75,2.68)
Model 2	0.77 (-4.56,6.10)	-0.63 (-6.17,4.91)	0.07 (-3.37,3.50)	0.56 (-2.09,3.21)
CEL				
Model 1	-3.00 (-6.51,0.50)	-1.24 (-4.80,2.32)	-0.54 (-2.80,1.72)	0.20 (-1.49,1.90)
Model 2	-3.67 (-7.86,0.53)	-3.69 (-8.05,0.70)	-2.51 (-5.21,0.20)	-0.42 (-2.51,1.68)
MG-H1				
Model 1	-2.42 (-5.91,1.07)	0.61 (-2.93,4.15)	-1.33 (-3.57,0.92)	0.24 (-1.44,1.93)
Model 2	-1.03 (-5.71,3.66)	-1.89 (-6.76,2.98)	-2.53 (-5.54,0.49)	0.27 (-2.06,2.60)
AGE z-score				
Model 1	-1.82 (-5.35,1.70)	0.53 (-3.05,4.12)	-0.25 (-2.52,2.02)	0.50 (-1.21,2.20)
Model 2	-1.94 (-6.84,2.97)	-2.70 (-7.80,2.40)	-2.17 (-5.33,0.99)	0.07 (-2.37,2.52)

Beta coefficients (B) and 95% CI represent the change in endothelial dysfunction biomarker (per µg/L for sICAM-1, sVCAM-1, and eSelectin, per % for vWf) per SD-change in dietary AGE intake. CML: N^{ε}-(carboxymethyl)lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. Note that higher concentrations of biomarkers indicate worse endothelial function.

Model 1: Adjusted for participant characteristics: age, sex, glucose metabolism status.

Model 2: Additionally adjusted for cardiovascular risk factors and lifestyle factors – waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, office systolic blood pressure, and use of antihypertensive medication, caloric intake, educational level, alcohol intake, and the Dutch Healthy Diet index.

Supplementary Table 4.10 Associations between dietary AGEs (Beta (95% CI)) and endothelial dysfunction plasma biomarkers with additional adjustment for microangiopathy

Dietary AGE (SD/day)	**Plasma Biomarker z-score** (SD)
CML	
Model 1	0.03 (-0.01,0.06)
Model 2	0.01 (-0.05,0.06)
Model 2a	-0.00 (-0.05,0.05)
CEL	
Model 1	-0.02 (-0.05,0.02)
Model 2	**-0.05 (-0.09,-0.01)**
Model 2a	**-0.05 (-0.09,-0.01)**
MG-H1	
Model 1	-0.01 (-0.05,0.02)
Model 2	-0.03 (-0.08,0.02)
Model 2a	-0.03 (-0.08,0.01)
AGE z-score	
Model 1	-0.00 (-0.04,0.03)
Model 2	-0.03 (-0.08,0.02)
Model 2a	-0.04 (-0.09,0.01)

Beta coefficients (B) and 95% CI represent the change in endothelial dysfunction biomarker (in SD) per SD-change in dietary AGE intake. Significant associations are shown in bold. CML: N^{ε}-(carboxymethyl)lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. ***n*** = 2836.

Model 1: Adjusted for participant characteristics: age, sex, glucose metabolism status.

Model 2: Additionally adjusted for cardiovascular risk factors and lifestyle factors: waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, systolic blood pressure, and use of antihypertensive medication, caloric intake, educational level, alcohol intake, and the Dutch Healthy Diet index.

Model 2a: Additionally adjusted for markers of microangiopathy: albuminuria, eGFR, history of CVD, retinopathy.

Supplementary Table 4.11 Associations between dietary AGEs (Beta (95% CI)) and endothelial dysfunction plasma biomarkers with additional adjustment for physical activity

Dietary AGE (SD/day)	**Plasma Biomarker Z-score** (SD)
CML	
Model 1	0.02 (-0.01,0.06)
Model 2	0.01 (-0.05,0.06)
Model 2b	0.01 (-0.05,0.07)
CEL	
Model 1	-0.01 (-0.05,0.03)
Model 2	-0.03 (-0.08,0.01)
Model 2b	-0.03 (-0.08,0.01)
MG-H1	
Model 1	-0.01 (-0.04,0.03)
Model 2	-0.01 (-0.06,0.04)
Model 2b	-0.01 (-0.06,0.04)
AGE z-score	
Model 1	0.00 (-0.04,0.04)
Model 2	-0.03 (-0.09,0.03)
Model 2b	-0.03 (-0.08,0.03)

Beta coefficients (B) and 95% CI represent the change in endothelial dysfunction biomarker (in SD) per SD-change in dietary AGE intake. Significant associations are shown in bold. CML: N^{ε}-(carboxymethyl)lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. *n* = 2405.

Model 1: Adjusted for participant characteristics: age, sex, glucose metabolism status.

Model 2: Additionally adjusted for cardiovascular risk factors and lifestyle factors: waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, systolic blood pressure, and use of antihypertensive medication, caloric intake, educational level, alcohol intake, and the Dutch Healthy Diet index.

Model 2b: Additionally adjusted for physical activity (Activpal)

Supplementary Table 4.12 Associations between dietary AGEs (Beta (95% CI)) and retinal flicker light-induced venular dilation response with further adjustment for physical activity.

Dietary AGE (SD/day)	**Flicker light-induced venular dilation** (%-change from baseline)
CML	
Model 1	0.05 (-0.06,0.15)
Model 2	0.11 (-0.05,0.28)
Model 2b	0.11 (-0.05,0.28)
CEL	
Model 1	0.09 (-0.02,0.20)
Model 2	0.13 (-0.00,0.26)
Model 2b	0.13 (-0.00,0.26)
MG-H1	
Model 1	0.03 (-0.07,0.14)
Model 2	0.06 (-0.08,0.20)
Model 2b	0.06 (-0.09,0.20)
AGE z-score	
Model 1	0.06 (-0.04,0.17)
Model 2	0.11 (-0.04,0.26)
Model 2b	0.11 (-0.05,0.26)

Beta coefficients (B) and 95% CI represent the change in flicker light-induced venular dilation (%-change over baseline) per SD-change in dietary AGE intake. CML: N^{ε}-(carboxymethyl)lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. *n* = 1817.

Model 1: Adjusted for participant characteristics: age, sex, glucose metabolism status.

Model 2: Additionally adjusted for cardiovascular risk factors and lifestyle factors: waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, systolic blood pressure, and use of antihypertensive medication, caloric intake, educational level, alcohol intake, and the Dutch Healthy Diet index.

Model 2b: Additionally adjusted for physical activity (Activpal)

Supplementary Table 4.13 Associations between dietary AGEs (Beta (95% CI)) and retinal flicker light-induced venular dilation response with further adjustment for microangiopathy.

Dietary AGE (SD/day)	**Flicker light-induced venular dilation** (%-change from baseline)
CML	
Model 1	0.02 (-0.08,0.12)
Model 2	0.12 (-0.03,0.28)
Model 2a	0.12 (-0.04,0.28)
CEL	
Model 1	0.07 (-0.03,0.17)
Model 2	**0.13 (0.01,0.25)**
Model 2a	**0.13 (0.01,0.25)**
MG-H1	
Model 1	0.01 (-0.09,0.11)
Model 2	0.06 (-0.08,0.19)
Model 2a	0.06 (-0.08,0.19)
AGE z-score	
Model 1	0.03 (-0.06,0.13)
Model 2	0.10 (-0.04,0.24)
Model 2b	0.11 (-0.03,0.25)

Beta coefficients (B) and 95% CI represent the change in flicker light-induced venular dilation (%-change over baseline) per SD-change in dietary AGE intake.CML: N^{ε}-(carboxymethyl)lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. *n* = 2074. Model 1: Adjusted for participant characteristics: age, sex, glucose metabolism status. Model 2: Additionally adjusted for cardiovascular risk factors and lifestyle factors: waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, systolic blood pressure, and use of antihypertensive medication, caloric intake, educational level, alcohol intake, and the Dutch Healthy Diet index. Model 2a: Additionally adjusted for markers of microangiopathy: albuminuria, eGFR, history of CVD, and retinopathy.

Supplementary Table 4.14 Associations between dietary CML and MG-H1 (Beta (95% CI)) and CRVE in subgroups of glucose metabolism status

Dietary AGE (SD/day)	**CRVE** (µm)	$P_{interaction}$
CML		
Total sample	-0.25 (-2.15,1.62)	
Normal glucose metabolism	0.10 (-2.14,1.63)	
Prediabetes	-3.42 (-7.56,0.72)	0.42
T2DM	0.23 (-3.17,3.63)	**0.03**
Other	18.35 (-39.99,76.70)	0.08
MG-H1		
Total sample	-0.78 (-2.440.88)	
Normal glucose metabolism	-0.53 (-3.06,2.01)	
Prediabetes	**-3.39 (-6.62,-0.15)**	0.46
T2DM	0.86 (-2.21,3.94)	**0.02**
Other	21.79 (-12.23,55.81)	0.14

Beta coefficients (B) and 95% CI represent the change CRVE (in um) per SD-change in dietary AGE intake. Significant associations are shown in bold. CML: N^{ε}-(carboxymethyl)lysine. CEL: N^{ε}-(1-carboxyethyl)lysine. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. Other types of diabetes: Type 1 diabetes or steroid/surgery-induced diabetes. Associations are adjusted for participant characteristics: age, sex, glucose metabolism status; cardiovascular risk factors – waist circumference, total/high-density lipoprotein ratio, triglycerides, smoking habits, use of lipid-lowering medication, systolic blood pressure, and use of antihypertensive medication; lifestyle factors: caloric intake, educational level, alcohol intake and the Dutch Healthy Diet index.

dietary AGEs in CEREALS (CRUNCHY MUESLY)
CML: 0.5; CEL 0.5; MG-H1: 8.6 mg/100g

Chapter 5

Habitual intake of AGEs is not associated with worse insulin sensitivity, worse beta-cell function, or presence of prediabetes or type 2 diabetes - The Maastricht Study

Armand M.A. Linkens, Simone J.M.P. Eussen, Alfons J.H.M. Houben, Andrea Mari, Pieter C Dagnelie, Coen D.A. Stehouwer, Casper G. Schalkwijk.

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Abstract

Background: A diet high in advanced glycation endproducts (AGEs) is a potential risk factor for insulin resistance, beta-cell dysfunction, and ultimately type 2 diabetes.

Objective: We investigated associations between habitual intake of dietary AGEs and glucose metabolism in a population-based setting.

Methods: In 6275 participants of The Maastricht Study (mean ± SD age: 60 ± 9, 15.1% prediabetes and 23.2% type 2 diabetes), we estimated habitual intake of dietary AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) by combining a validated food frequency questionnaire (FFQ) with our mass-spectrometry dietary AGE database. We determined insulin sensitivity (Matsuda- and HOMA-IR index), beta-cell function (C-peptidogenic index, glucose sensitivity, potentiation factor, and rate sensitivity), glucose metabolism status, fasting glucose, HbA1c, post-OGTT glucose, and OGTT glucose incremental area under the curve. Cross-sectional associations between habitual AGE intake and these outcomes were investigated using a combination of multiple linear regression and multinomial logistic regression adjusting for several potential confounders (demographic, cardiovascular, and lifestyle factors).

Results: Generally, higher habitual intake of AGEs was not associated with worse indices of glucose metabolism, nor with increased presence of prediabetes or type 2 diabetes. Higher dietary MG-H1 was associated with better beta-cell glucose sensitivity.

Conclusions: The present study does not support an association of dietary AGEs with impaired glucose metabolism. These findings should be validated in large, prospective cohort studies.

Introduction

Advanced glycation endproducts (AGEs) are a heterogeneous group of bioactive compounds, formed in vivo from the non-enzymatic reaction between sugars and amino groups in proteins and other amino acid containing macromolecules[1]. AGE formation is a naturally occurring process but is accelerated by hyperglycemia and oxidative stress, such as occurs in type 2 diabetes[2]. AGEs are well known contributors to hyperglycemia-associated vascular complications in diabetes[3-9]. Importantly, AGEs may also contribute to development of type 2 diabetes by influencing insulin sensitivity[6, 10, 11] and insulin secretion[12-14].

In addition to endogenous formation, AGEs are also widely present in foods, especially those exposed to dry heat[15]. Animal[16] and human studies[15, 17] indicate that these dietary AGEs are absorbed into the circulation. As such, AGEs in food are a potential risk factor for the development of type 2 diabetes. The potential of a low AGE diet to improve insulin sensitivity and secretion has been investigated in two well-controlled randomized-controlled trials (RCT)[17, 18]. De Courten et al. found an improvement in insulin sensitivity, but not in insulin secretion, in 20 healthy but overweight individuals after a 2-week diet low compared to high in AGEs in a cross-over design[18]. In contrast, we recently found no difference in insulin sensitivity, secretion, and clearance in 72 abdominally obese individuals after a 4-week diet low compared to high in AGEs in a parallel design[17]. However, associations between AGE intake from the habitual diet and glucose metabolism have not yet been investigated in the general population.

Therefore, we examined for the first time whether higher habitual intake of dietary AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) is associated with greater odds of prediabetes and type 2 diabetes, greater insulin resistance, worse beta-cell function, and worse measures of hyperglycemia in a population-based cohort.

Material & Methods

Study design and population

We used data from The Maastricht Study, an observational prospective population-based cohort study. The rationale and methodology have been described previously[19]. In brief, the study focuses on the etiology, pathophysiology, complications, and comorbidities of type 2 diabetes and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Participants were recruited

through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. Recruitment was stratified according to known diabetes status, with an oversampling of individuals with type 2 diabetes, for reasons of efficiency. We have used cross-sectional data from the first 7689 participants, who completed the baseline survey between November 2010 and December 2017. The examinations of each participant were performed within a time window of three months. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

Food intake and dietary advanced glycation endproducts

We assessed dietary intake by a validated 253-item food frequency questionnaire (FFQ)[20]. This FFQ contains 101 questions on consumption with a reference period of one year. The FFQ collected information on the intake of major food groups. All participants filled out the FFQ after their first visit to the study center.

Food intake was determined by the combination of frequency questions with quantity questions. For the frequency questions, 11 options were available ranging from "not used" to 7 days/week. For the quantity questions, variable options were available based on fourteen standard household servings, ranging from < 1/day to > 12/day. Average daily consumption of food items was then calculated by multiplying the frequency and amount. Energy and nutrient intakes were subsequently determined by transcribing food items into food codes embedded in the Dutch Food Composition Table 2011[21]. Additionally, we determined the Dutch Healthy Diet (DHD) index based on this food intake data. The DHD-index is a measure of diet quality as it assesses adherence to the Dutch dietary guidelines[22]. A higher index has been associated with more nutrient-dense diets and lower risk of mortality[23, 24]. In our statistical analyses we used a modified version of the DHD-index that does not contain filtered coffee consumption as this information was not collected by the FFQ, and alcohol intake was not included as we already adjusted for alcohol intake as an individual variable.

Dietary AGE intake was determined by coupling the consumption of food items within the FFQ to our dietary AGE database[25]. In this database, three major AGEs, CML, CEL, and MG-H1, were quantified in protein fractions of food products using highly specific ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). In total, this database includes over 200 food products commonly consumed in a Western diet. For each participant, AGE intake was estimated as described previously[15]. Some of the food products in the FFQ were not analyzed for AGEs content. AGE content of these specific products were estimated by

matching them to other products that were comparable in macronutrient profile and preparation method. Only after completion of the FFQ, participants were informed about their glucose metabolism status. As a result, estimation of dietary AGEs was not affected by a potential change in dietary habits upon dietary advice from health care workers in case participants were newly diagnosed with type 2 diabetes.

Glucose metabolism status

To determine glucose metabolism status, all participants (except those who used insulin) underwent a standardized 7-point oral glucose tolerance test (OGTT) after an overnight fast. Blood samples were taken at baseline and at 15, 30, 45, 60, 90, and 120 minutes after ingestion of a 75 g glucose drink. For safety reasons, participants with a fasting glucose level above 11.0 mmol/L, as determined by a finger prick, did not undergo the oral glucose tolerance test. For these individuals, fasting glucose level and information about glucose-lowering medication were used to determine glucose metabolism status. Glucose metabolism was classified according to the WHO 2006 criteria into normal glucose tolerance, impaired fasting glucose, impaired glucose tolerance, and type 2 diabetes[26]. For this study, we defined having either impaired fasting glucose or impaired glucose tolerance as prediabetes. Detailed information on collection and measurement of plasma glucose, insulin, and c-peptide are described in the Supplementary methods.

Insulin sensitivity

Insulin sensitivity was determined from glucose and insulin levels during the OGTT using the Matsuda index. The Matsuda index reflects whole body insulin sensitivity and is calculated as described previously[27]. Additionally, insulin sensitivity was determined from fasting glucose and insulin levels using the HOMA-IR2 index[28].

Beta-cell function

As beta-cell function (BCF) consists of multiple components, we used three mathematical model-based parameters (beta-cell glucose sensitivity, the beta-cell potentiation, and beta-cell rate sensitivity)[29], and a classic, relatively simple BCF-index (C-peptidogenic index)[30].

The simple C-peptidogenic index, also termed the insulinogenic index, reflects early phase insulin secretion and has a good ability to discriminate between normal glucose metabolism (NGM), prediabetes, and type 2 diabetes[30] and is an independent predictor of type 2 diabetes[30]. It is calculated as described previously[30].

The mathematical model parameter "beta-cell glucose sensitivity" is the slope of the glucose-insulin secretion dose-response function[29], and represents the dependence of insulin secretion on absolute glucose concentration at any time point during the OGTT. Beta-cell glucose sensitivity is a sensitive index to quantify beta-cell dysfunction[31]. The dose-response relationship is modulated by beta-cell potentiation, which accounts for higher insulin secretion during the descending phase of hyperglycemia than during the ascending phase of an OGTT for the same glucose concentration. Beta-cell potentiation is set as a positive function of time and averages 1 during the OGTT. Therefore, it represents the relative potentiation of the insulin secretion response to glucose. The beta-cell potentiation parameter used in the present analysis represents the ratio of the beta-cell potentiation factor at the end of the 2-h OGTT relative to the beta-cell potentiation factor at the start. "Beta-cell rate sensitivity" is a marker of early phase insulin release, and represents the dynamic dependence of insulin secretion on the rate of change in glucose concentration[29].

Measures of hyperglycemia

Fasting plasma glucose, HbA1c, glucose tolerance during the 2-hour OGTT, and plasma glucose after the 2-hour OGTT were used as additional measures of hyperglycemia[19]. Glucose tolerance was defined as the incremental area under the curve (iAUC) of plasma glucose during the OGTT relative to fasting glucose. The glucose iAUC was only calculated for individuals for which glucose at all time points were available.

Covariates

Smoking status and history of cardiovascular disease (CVD) were assessed by a questionnaire. Smoking status was categorized into never, former and current smoker. Waist circumference, total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), and triglycerides were determined as described elsewhere[19]. Estimated glomerular filtration rate (eGFR) was computed with the Chronic Kidney Disease Epidemiology Collaboration formula, using serum creatinine and cystatin C[32]. Information on the use of lipid-modifying, antihypertensive medication, and/or glucose-lowering medication that is generic names, doses and frequencies, were collected during an in person medication interview.

Statistical methods

Analyses were conducted using SPSS version 25 for Windows (IBM Corporation, Armonk, NY, USA). *p* values of < 0.05 were considered statistically significant. Baseline characteristics are shown for the total sample, and stratified by a dietary AGE z-score that represents an individuals' overall AGE intake. Because we measured three AGEs in food items which differ in abundance, we first calculated z-scores for CML, CEL and MG-H1, which were then averaged into a single dietary AGE z-score. Complete case analyses were performed for all outcomes. Differences in characteristics across quartiles of dietary AGE intake, as well as between participants who were excluded from the analyses because of missing covariates were tested with a one-way ANOVA and Kruskal-Wallis test for normally and non-normally divided variables, and with a chi-squared test for proportions.

We performed multiple linear regression to investigate the association between each standardized dietary AGE and standardized measurements of glucose metabolism. To attain normal distributions, the Matsuda- and HOMA-IR index were log-transformed. We performed multinomial logistic regression analyses to investigate the association between each standardized dietary AGE and glucose metabolism status (i.e. presence of prediabetes or type 2 diabetes).

To check whether associations between dietary AGEs and outcomes deviated from linearity, regression models using dietary AGEs as continuous and categorical exposures were compared with a likelihood ratio test. For the outcomes of beta-cell glucose sensitivity, rate sensitivity, and HbA1c, entering dietary AGEs as categorical exposures provided a statistically significant better fit. For these analyses, dietary AGEs were transformed into quartiles with the lowest quartile as a reference. Associations for continuous outcomes were presented as standardized betas (β) (95% CI) and as odds ratios (OR) (95% CI) for categorical outcomes.

Two models were fitted to adjust for potential confounding. In the semi-adjusted model, we adjusted for age (years) and sex (male/female). In the fully-adjusted model, we additionally adjusted for cardiovascular risk factors and lifestyle factors: history of CVD (%-yes), waist circumference (cm), systolic blood pressure (mmHg), triglycerides (mmol/L), LDL (mmol/L), total cholesterol/HDL ratio, use of antihypertensive- or lipid-modifying medication (%-yes), smoking status (former, current, never), estimated glomerular filtration rate (ml/min/1.73m^2), total energy intake (kcal/day), alcohol intake (g/day), the Dutch healthy diet index, physical activity (hours/week), and educational level (low/middle/high). In analyses were beta-cell function measures were outcomes, model 2 was additionally adjusted for insulin sensitivity expressed as the Matsuda index.

We also performed several sensitivity analyses. First, when considering type 2 diabetes as the outcome variable, we performed analyses in only the newly diagnosed type 2 diabetes and in the entire type 2 diabetes population to control for potential information bias regarding altered food intake after diagnosis of type 2 diabetes. Second, to address the oversampling of participants with type 2 diabetes in The Maastricht Study, we also performed all our analyses while excluding those with previously known type 2 diabetes. Third, we substituted BMI for waist circumference in the fully adjusted model, as another reflection of obesity. Fourth, we further investigated potential confounding by physical activity and socio-economic status by adjusting for accelerometer data and equivalent income instead of the CHAMPS physical activity questionnaire and educational level. These alternative methods are considered more accurate but have more missing values. As selection bias may occur in these analyses in subpopulations, we also performed our original analyses in these subpopulations. Fifth, as not all assumptions for linear regression were perfectly met, and transformation was not possible without data manipulation, we also transformed the outcomes of Matsuda index and indices of beta-cell function into tertiles, and investigated associations with dietary AGEs using multinomial logistic regression analysis.

We also performed several interaction analyses. For all outcomes, we tested for effect modification by age, sex, and estimated glomerular filtration rate by adding these variables as interaction terms into the fully adjusted models. The kidneys are responsible for clearing AGEs from plasma, and plasma AGEs are increased in those with kidney disease[33].

Results

Population characteristics

The selection of participants for the different outcomes are shown in Figure 5.1. Characteristics of the largest sample, those for the analyses of dietary AGEs and presence of prediabetes and type 2 diabetes, are shown in Table 5.1. Mean daily intake of AGEs was 3.6 ± 1.3 mg/day for CML, 3.2 ± 1.5 mg/day for CEL, and 24.9 ± 8.9 mg/day for MG-H1. Individuals with highest habitual intake of dietary AGEs were more likely to be more physically active, male, and consumed more, but slightly unhealthier food. Visually, these participants were less likely to have type 2 diabetes and to use glucose-lowering medication, but these comparisons did not reach statistical significance ($p = 0.096$ and $p = 0.092$, respectively)

Individuals that were excluded from analyses due to missing covariates had a worse cardiovascular risk profile in general, including higher prevalence of type 2 diabetes and subsequently higher fasting glucose and HbA1c, a trend for lower Matsuda index ($p = 0.085$) but lower HOMA-IR index, lower beta-cell glucose sensitivity, and more medication use ($p < 0.05$, Supplementary Table 5.1).

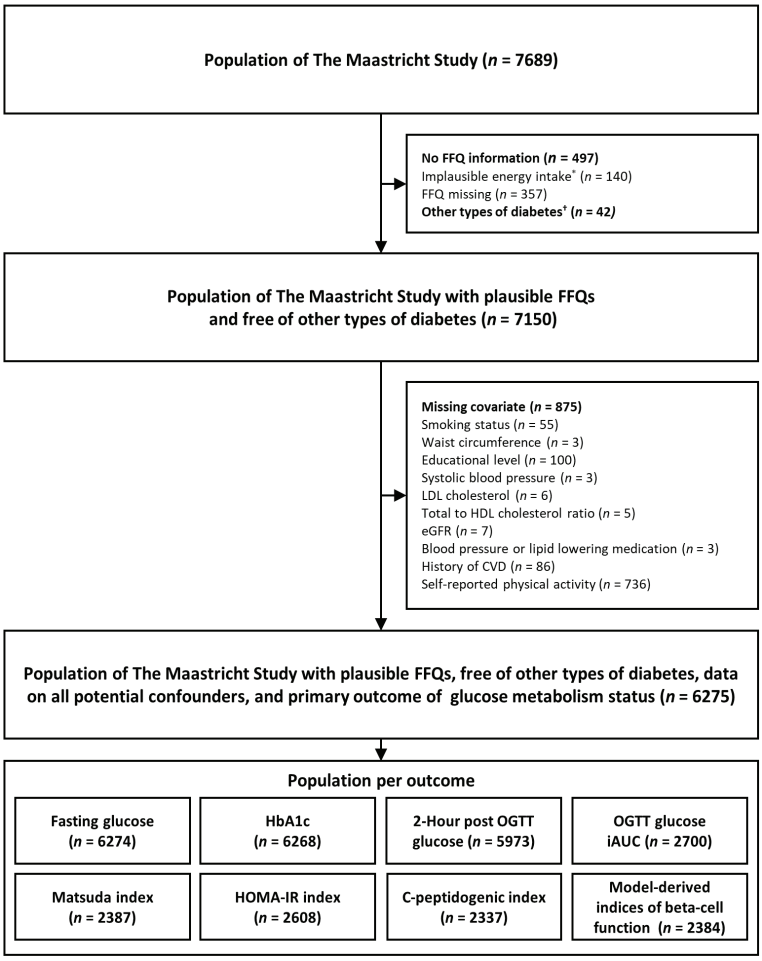

Figure 5.1 Flow chart of participant selection for the different outcomes. Please note that missing variables do not necessarily add up as they are not mutually exclusive, e.g. an individual may have missing information on both smoking status and educational level.
*Implausible energy intake: < 500 kcal or > 3500 kcal for women, < 800 or > 4000 kcal for men.
†Other types of diabetes: Type 1 diabetes mellitus, surgery-induced diabetes.
Abbreviations: FFQ: food frequency questionnaire. eGFR: estimated glomerular filtration rate. OGTT: oral glucose tolerance test.

Dietary AGEs and insulin sensitivity.

Habitual intake of dietary AGEs CML, CEL, and MG-H1 was not associated with the Matsuda index, both after adjustment for age- and sex, as well as after further adjustment for cardiovascular and lifestyle factors (Figure 5.2). Greater habitual intake of CML was associated with higher HOMA-IR index, but the association did not reach statistical significance in both the age- and sex-adjusted- and the fully adjusted model, with β (SD) and (95% CI) of 0.04 (-0.00,0.09) for the latter (Figure 5.2). Likewise, habitual intake of CEL and MG-H1 was also not associated with HOMA-IR in fully adjusted models.

Table 5.1 Baseline characteristics of participants of the Maastricht Study with information on all potential confounders, stratified by habitual dietary AGE intake

Characteristic	Total population (*n* = 6275)	Dietary AGE intake (z-score)			
		Q1 (*n* = 1568)	**Q2** (*n* = 1568)	**Q3** (*n* = 1568)	**Q4** (*n* = 1568)
Demographics					
Age (years)	59.8 ± 8.6	60.8 ± 8.5	60.1 ± 8.7	59.7 ± 8.5	58.6 ± 8.5
Sex (% male)	49.9	34.4	46.1	53.9	65.2
Lifestyle					
Smoking (%)					
Never	37.9	37.0	37.3	37.5	39.8
Former	50.2	50.1	50.9	51.4	48.5
Current	11.9	12.9	11.9	11.1	11.7
Waist circumference (cm)					
Males	100.7 ± 11.9	102.3 ± 11.9	101.1 ± 12.0	99.8 ± 11.2	100.4 ± 12.2
Females	89.2 ± 12.6	89.4 ± 13.1	88.2 ± 12.0	89.4 ±12.6	90.2 ± 12.8
BMI (kg/m^2)	26.9 ± 4.4	27.0 ± 4.6	26.6 ± 4.3	26.8 ± 4.2	27.1 ± 4.5
Physical activity (h/week)	13.9 ± 8.2	13.5 ± 7.9	14.1 ± 8.2	13.9 ± 7.9	14.4 ± 8.5
Education (%)					
Low	33.4	38.4	31.5	32.7	31.0
Medium	27.9	24.4	28.0	30.0	29.3
High	38.7	37.2	40.5	37.3	39.8
Biological					
Office Systolic blood pressure (mmHg)	133.6 ± 17.8	133.0 ± 18.1	133.0 ± 17.8	134.5 ± 18.1	133.8 ±16.9
Office Diastolic blood pressure (mmHg)	75.6 ± 9.8	74.6 ± 9.5	75.2 ± 9.7	76.2 ± 10.0	76.2 ± 9.7
LDL cholesterol (mmol/L)	3.1 ± 1.0	3.0 ± 1.0	3.0 ± 1.0	3.1 ± 1.0	3.1 ± 1.0
Total-to-HDL cholesterol ratio	3.6 ± 1.2	3.5 ± 1.1	3.5 ± 1.2	3.7 ± 1.2	3.8 ± 1.2
Triglycerides (mmol/L)	1.4 ± 0.8	1.4 ± 0.8	1.4 ± 0.8	1.4 ± 0.8	1.4 ± 0.9
eGFR (mL/min/1.73m^2)	82.0 ± 14.0	81.5 ± 14.8	81.5 ± 13.7	81.8 ± 13.8	83.4 ± 13.6
Medical history of CVD (% yes)	16.2	15.8	17.3	16.8	14.9
Medication use					
Glucose-lowering medication (% yes)	16.8	18.0	17.0	16.6	15.7
Anti-hypertensive medication (% yes)	37.0	39.8	36.0	38.0	34.4
Lipid-lowering medication (% yes)	31.3	32.8	32.4	31.0	29.2
Dietary intake					
Energy intake (kJ/day)	8956 ± 2487	6674 ± 1578	8273 ± 1470	9487 ± 1754	11388 ± 2269
Alcohol intake (g/day)	8.2 [1.6,18.3]	7.4 [0.8,18.1]	7.5 [1.5,17.1]	8.5 [2.0,17.8]	9.2 [2.6,20.1]
Dutch Healthy Diet Index[*]	76.6 ± 14.5	78.8 ± 14.3	78.4 ± 14.4	75.8 ± 14.1	73.4 ± 14.6
Dietary CML (mg/day)	3.6 ± 1.3	2.3 ± 0.5	3.2 ± 0.4	3.9 ± 0.5	5.3 ± 1.2
Dietary CEL (mg/day)	3.2 ± 1.5	1.9 ± 0.4	2.6 ± 0.4	3.3 ± 0.5	5.0 ± 1.7
Dietary MG-H1 (mg/day)	24.9 ± 8.9	15.9 ± 3.1	21.5 ± 2.6	26.2 ± 3.0	35.9 ± 8.9
Glucose metabolism					
Glucose metabolism status (%)					
Normal glucose metabolism	61.8	59.2	62.8	61.5	63.7
Prediabetes	15.1	15.6	13.8	16.4	14.4
Type 2 diabetes	23.2	25.3	23.4	22.1	21.9
Fasting glucose (mmol/L) [†]	5.9 ± 1.6	5.9 ± 1.5	5.8 ± 1.4	5.9 ± 1.5	5.9 ± 1.6
HbA1c (%)[‡]	5.7 ± 0.8	5.8 ± 0.8	5.7 ± 0.8	5.7 ± 0.8	5.8 ± 0.9
2-hours post OGTT glucose [§] (mmol/L)	7.5 ± 4.0	7.8 ± 4.2	7.6 ± 4.1	7.4 ± 3.8	7.3 ± 3.9
OGTT glucose iAUC [‖] (mmol/L·min)	348 ± 274	365 ± 290	333 ± 280	347 ± 280	348 ± 274
HOMA-IR index [¶]	1.4 [1.0,2.1]	1.4 [0.9,2.0]	1.3 [0.9,2.0]	1.4 [1.0,2.1]	1.4 [1.0,2.3]
Matsuda index [#]	3.5 [2.1,5.3]	3.4 [2.1,5.5]	3.7 [2.3,5.4]	3.6 [1.9,5.2]	3.3 [2.0,5.1]
C-peptidogenic index [**]	465 ± 1165	533 ± 1216	438 ± 1341	428 ± 1307	464 ± 671
Glucose sensitivity [††] (pmol/min/m^2/mmol/L)	84.0 ± 55.6	80.6 ± 57.1	85.5 ± 55.0	86.1 ± 55.0	83.7 ± 55,6
Potentiation factor [††]	1.6 ± 0.7	1.7 ± 0.7	1.6 ± 0.7	1.6 ± 0.7	1.6 ± 0.7
Rate sensitivity[9] (pmol/m^2/mmol/L)	758 ± 1000	710 ± 876	743 ± 785	837 ± 1396	746 ± 852

Data are presented as means ± SD, medians [interquartile range], or percentages. Differences in characteristics across quartiles of dietary AGE intake were tested with a one-way ANOVA and Kruskal-Wallis test for normally and non-normally divided variables, and with a chi-squared test for proportions. [*]Modified version of the Dutch Healthy diet index that does not include alcohol intake. [†]Fasting glucose available in *n* = 6274. [‡]HbA1c available in *n* = 6268. [§]2-hours post OGTT glucose available in *n* = 5973. [‖]OGTT glucose iAUC available in *n* = 2700. [¶]HOMA-IR index available in *n* = 2608. [#]Matsuda index available in *n* = 2387. [**]C-peptidogenic index available in *n* = 2393. [††]Mathematically-modelled indices of beta-cell function available in *n* = 2440. Abbreviations: CEL: N$^{\varepsilon}$-(1-carboxyethyl)lysine. CML: N$^{\varepsilon}$-(carboxymethyl)lysine. eGFR: estimated glomerular filtration rate. iAUC: incremental area under the curve. MG-H1: N$^{\delta}$-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. OGTT: oral glucose tolerance test.

Dietary AGEs and beta-cell function

Habitual intake of dietary AGEs CML, CEL, and MG-H1 was not associated with the C-peptidogenic index or beta-cell potentiation factor, both after adjustment for age- and sex, as well as after further adjustment for cardiovascular and lifestyle factors (Figure 5.2). In contrast, habitual intake of dietary AGEs, especially MG-H1, were non-linearly associated with beta-cell glucose sensitivity. After full adjustment for potential confounders, beta-cell glucose sensitivity was higher among those in Q2, Q3, and Q4 of MG-H1 intake than among those in Q1, with β (SD) and (95% CI) for Q2 of 0.15 (0.04,0.26), for Q3 of 0.13 (0.01,0.25), and for Q4 of 0.21 (0.07,0.36) (Table 5.2). Although Q2 and Q3 of CML intake were also associated with higher beta-cell glucose sensitivity in the age- and sex-adjusted model, these associations were attenuated and lost statistical significance after full adjustment for potential confounders (Table 5.2). For CEL, only Q3 was associated with higher beta-cell glucose sensitivity after full adjustment for potential confounders, with β (SD) and (95% CI) of 0.14 (0.02,0.25) (Table 5.2).

Beta-cell rate sensitivity was not different between quartiles of CEL and MG-H1 intake in the fully adjusted models (Table 5.2). Only in those in Q3 of CML intake, we observed higher beta-cell rate sensitivity in the fully adjusted model, with β (SD) and (95% CI) of 0.18 (0.05,0.31) (Table 5.2).

Dietary AGEs and measures of hyperglycemia

Dietary CML and CEL were not associated with fasting plasma glucose, 2-hours post-OGTT glucose, and OGTT glucose iAUC in the fully adjusted models (Figure 5.2). After adjustment for age and sex, higher intake of dietary MG-H1 was associated with marginally lower fasting plasma glucose, 2-hours post-OGTT glucose, and OGTT glucose iAUC, but these associations were attenuated and lost statistical significance after further adjustment for cardiovascular and lifestyle factors (Figure 5.2).

For HbA1c, we observed opposing linear and non-linear associations between dietary AGEs. In the fully adjusted model, those among Q2 of CML intake and Q4 of CEL intake had lower HbA1c compared to those among Q1, with β (SD) and (95% CI) of-0.07 (-0.13,-0.01) and-0.09 (-0.16,-0.02), respectively (Table 5.2). Additionally, for quartiles of CEL intake, a negative linear trend for HbA1c was observed (*p* trend = 0.021). In contrast, greater intake of MG-H1 was associated with marginally higher HbA1c in the fully adjusted model, with β (SD) and (95% CI) of 0.03 (0.00,0.06) (Table 5.2).

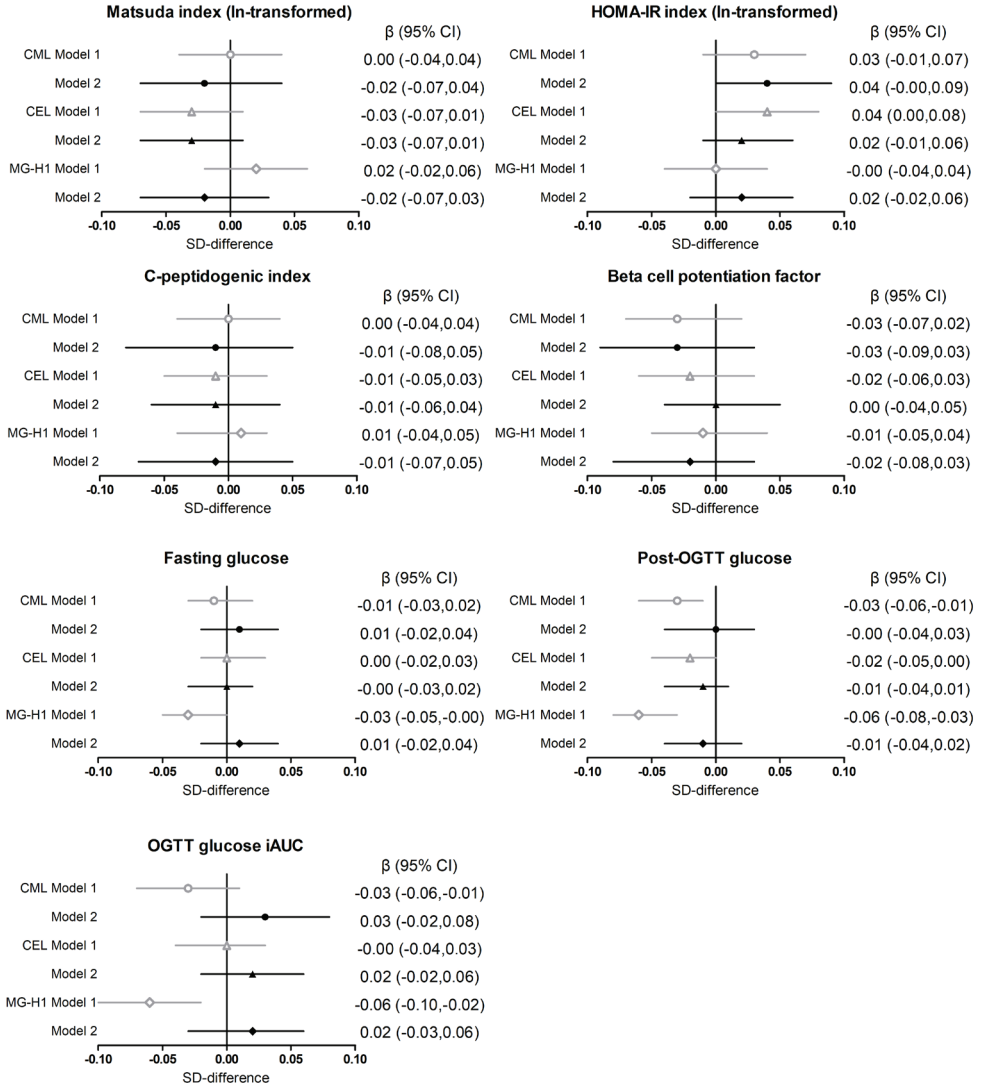

Figure 5.2 Multivariate-adjusted associations of dietary AGEs (in SD/day) and glucose measurements tested with multiple linear regression analysis. Beta's and their (95% CIs) indicate the difference in standardized glucose measurement per 1 SD difference in dietary AGE intake. Sample sizes: Matsuda index *n* = 2387, HOMA-IR index *n* = 2608. C-peptidogenic index *n* = 2337, Beta-cell potentiation factor *n* = 2384, fasting glucose *n* = 6274, Post-OGTT glucose *n* = 5973, and OGTT glucose iAUC *n* = 2700.

Semi-adjusted model 1: Adjusted for age and sex.

Fully-adjusted model 2: Additionally adjusted for cardiovascular risk factors (prior CVD, waist circumference, office systolic blood pressure, triglycerides, LDL-cholesterol, total/high-density lipoprotein ratio, use of lipid-lowering medication or antihypertensive medication, smoking habits, and kidney function) and lifestyle factors (energy intake, alcohol intake, the Dutch Healthy Diet index, physical activity, and educational level).

Table 5.2 (Nonlinear) associations between dietary AGEs and glucose metabolism outcomes in adults of The Maastricht Study.

Outcome	Dietary AGE β (95% CI)	Continuous (SD/day)	Quartiles of AGE intake				P_{trend}
			Q1 (ref)	**Q2**	**Q3**	**Q4**	
Beta-cell glucose sensitivity	**CML**		< 2.7 mg/day	2.7-3.5 mg/day	3.5-4.4 mg/day	>4.4 mg/day	
	Semi-adjusted*	0.03 (-0.01,0.07)	0	**0.12 (0.00,0.23)**	**0.17 (0.05,0.28)**	0.05 (-0.07,0.16)	0.501
	Fully-adjusted†	0.02 (-0.04,0.08)	0	0.07 (-0.04,0.18)	0.12 (-0.01,0.24)	0.01 (-0.14,0.16)	0.963
	CEL		< 2.3 mg/day	2.3-2.9 mg/day	2.9-3.8 mg/day	>3.8 mg/day	
	Semi-adjusted*	0.03 (-0.02,0.07)	0	0.02 (-0.09,0.13)	**0.17 (0.06,0.28)**	0.06 (-0.05,0.18)	0.169
	Fully-adjusted†	0.01 (-0.04,0.05)	0	0.01 (-0.10,0.12)	**0.14 (0.02,0.25)**	0.05 (-0.08,0.18)	0.381
	MG-H1		< 19.1 mg/day	19.1-23.9 mg/day	23.9-29.8 mg/day	>29.8 mg/day	
	Semi-adjusted*	0.04 (-0.00,0.08)	0	**0.16 (0.05,0.27)**	0.10 (-0.10,0.22)	**0.18 (0.06,0.29)**	**0.012**
	Fully-adjusted†	0.04 (-0.02,0.09)	0	**0.15 (0.04.0.26)**	**0.13 (0.01,0.25)**	**0.21 (0.07,0.36)**	**0.013**
Beta-cell rate sensitivity	**CML**		< 2.7 mg/day	2.7-3.5 mg/day	3.5-4.4 mg/day	>4.4 mg/day	
	Semi-adjusted*	0.02 (-0.02,0.06)	0	-0.02 (-0.14,0.09)	**0.21 (0.10,0.33)**	0.00 (-0.11,0.12)	0.465
	Fully-adjusted†	0.01 (-0.05,0.07)	0	-0.06 (-0.17,0.06)	**0.18 (0.05,0.31)**	-0.02 (-0.18,0.14)	0.790
	CEL		< 2.3 mg/day	2.3-2.9 mg/day	2.9-3.8 mg/day	>3.8 mg/day	
	Semi-adjusted*	0.02 (-0.03,0.06)	0	0.09 (-0.02,0.21)	0.11 (-0.01,0.22)	0.06 (-0.06,0.18)	0.447
	Fully-adjusted†	0.01 (-0.04,0.06)	0	0.08 (-0.03,0.20)	0.09 (-0.03,0.21)	0.06 (-0.08,0.20)	0.618
	MG-H1		< 19.1 mg/day	19.1-23.9 mg/day	23.9-29.8 mg/day	>29.8 mg/day	
	Semi-adjusted*	0.03 (-0.02,0.07)	0	0.09 (-0.03,0.20)	**0.13 (0.02,0.24)**	0.07 (-0.05,0.18)	0.285
	Fully-adjusted†	0.01 (-0.05,0.06)	0	0.06 (-0.06,0.18)	0.11 (-0.02,0.24)	0.02 (-0.14,0.18)	0.835
HbA1c	**CML**		< 2.7 mg/day	2.7-3.5 mg/day	3.5-4.4 mg/day	>4.4 mg/day	
	Semi-adjusted*	0.02 (-0.01,0.05)	0	**-0.10 (-0.16,-0.03)**	-0.06 (-0.13,0.01)	-0.00 (-0.07,0.07)	0.571
	Fully-adjusted†	0.00 (-0.03,0.04)	0	**-0.07 (-0.13,-0.01)**	-0.06 (-0.13,0.01)	-0.02 (-0.11,0.06)	0.785
	CEL		< 2.3 mg/day	2.3-2.9 mg/day	2.9-3.8 mg/day	>3.8 mg/day	
	Semi-adjusted*	0.01 (-0.01,0.04)	0	-0.04 (-0.11,0.03)	-0.01 (-0.08,0.06)	-0.04 (-0.11,0.03)	0.482
	Fully-adjusted†	-0.01 (-0.03,0.02)	0	-0.05 (-0.11,0.01)	-0.06 (-0.12,0.01)	**-0.09 (-0.16,-0.02)**	**0.021**
	MG-H1		< 19.0 mg/day	19.0-23.7 mg/day	23.7-29.3 mg/day	>29.3 mg/day	
	Semi-adjusted*	0.01 (-0.01,0.04)	0	-0.04 (-0.11,0.03)	-0.02 (-0.09,0.05)	-0.04 (-0.11,0.03)	0.421
	Fully-adjusted†	**0.03 (0.00,0.06)**	0	-0.01 (-0.07,0.06)	0.01 (-0.06,0.07)	0.00 (-0.08,0.08)	0.884

Multivariate-adjusted associations of dietary AGEs and glucose measurements tested with multiple linear regression analysis. Standardized beta's and their 95% CIs indicate the difference in standardized glucose measurement per 1 SD difference in dietary AGE intake or per quartile of dietary AGE intake compared to the first reference quartile. Sample sizes: Beta-cell glucose and rate sensitivity *n* = 2384, quartile size *n* = 596, HbA1c *n* = 6268, quartile size *n* = 1567. Statistically significant values are shown bold.

*Semi-adjusted model: Adjusted for age and sex. †Fully-adjusted model: Additionally adjusted for cardiovascular risk factors (prior CVD, waist circumference, office systolic blood pressure, triglycerides, LDL-cholesterol, total/high-density lipoprotein ratio, use of lipid-lowering medication or antihypertensive medication, smoking habits, and kidney function) and lifestyle factors (energy intake, alcohol intake, the Dutch Healthy Diet index, physical activity, and educational level).

Dietary AGEs and presence of prediabetes and type 2 diabetes

Habitual intake of dietary CML, CEL, and MG-H1 was not statistically different between individuals with NGM, prediabetes, or type 2 diabetes ($p > 0.05$, data not shown). Intake of dietary CML, CEL, and MG-H1 were not associated with presence of prediabetes (Table 5.3). While adjusting for age and sex, higher intake of dietary CML and MG-H1 were associated with lower presence of type 2 diabetes: OR (95% CI) of 0.93 (0.87,0.99) and 0.88 (0.82,0.94), respectively (Table 5.3). However, these associations were attenuated and lost statistical significance after further adjustment for cardiovascular and lifestyle factors: OR (95% CI) of 0.99 (0.88,1.11) and 0.98 (0.88,1.09). Intake of dietary CEL was not associated with presence of type 2 diabetes in any of the models. We additionally investigated the association between habitual intake of dietary AGEs and presence of newly diagnosed type 2 diabetes (*n* = 265), and results were essentially similar (data not shown).

Table 5.3 Associations between dietary AGEs and glucose metabolism status in adults of The Maastricht Study

Dietary AGE (SD/day)	**NGM** (*n* = 3877)	**Prediabetes** (*n* = 945)	**T2DM** (*n* = 1453)
CML	Reference		
Semi-adjusted OR*	1	0.97 (0.90,1.04)	**0.93 (0.87,0.99)**
Fully-adjusted OR†	1	0.99 (0.88,1.11)	0.99 (0.88,1.11)
CEL	Reference		
Semi-adjusted OR*	1	1.02 (0.95,1.09)	0.96 (0.89,1.02)
Fully-adjusted OR†	1	1.01 (0.93,1.11)	0.97 (0.88,1.06)
MG-H1	Reference		
Semi-adjusted OR*	1	0.94 (0.87,1.01)	**0.88 (0.82,0.94)**
Fully-adjusted OR†	1	0.98 (0.88,1.10)	0.98 (0.88,1.09)

Odds ratios (OR) and 95% CIs represent the likelihood of having prediabetes or T2DM per 1-SD change in dietary AGE intake. Abbreviations: AGE, advanced glycation endproduct; N^{ε}-(1-carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl) lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

* Adjusted for age and sex.

† Additionally adjusted for cardiovascular risk factors (prior CVD, waist circumference, office systolic blood pressure, triglycerides, LDL-cholesterol, total/high-density lipoprotein ratio, use of lipid-lowering medication or antihypertensive medication, smoking habits, and kidney function) and lifestyle factors (energy intake, alcohol intake, the Dutch Healthy Diet index, physical activity, and educational level).

Interaction analyses

There were statistically significant interactions for the associations between dietary AGEs and Matsuda index by kidney function (all *p* for interactions < 0.005). As such, we stratified our analyses by kidney function above or below 60 ml/min/1.73m^2. In those with kidney function below 60 ml/min/1.73m^2 (*n* = 111, median eGFR [IQR]

of 55 [48,58]), higher intake of CEL was associated with lower Matsuda index, both in the age- and sex-adjusted model as in the fully adjusted model, with β (SD) and (95% CI) of -0.06 (-0.12,-0.00) and -0.07 (-0.14,-0.01), respectively (Supplementary Table 5.2). Results for other dietary AGEs remained materially unchanged. Similarly, interactions by kidney function were found for the associations between CML, MG-H1, and fasting plasma glucose and HbA1c (all *p* for interactions < 0.05). However, results were unchanged in fully adjusted models of stratified analyses (data not shown). For all other outcomes, there were no statistically significant interactions by age, sex, or kidney function.

Sensitivity analyses

In fully adjusted models in subpopulations with accelerometer data, higher intake of CEL was associated with lower Matsuda index while higher intake of CML was associated with higher HOMA-IR, both indicating higher insulin resistance. However, introducing physical activity into the model did not change the effect size for the association between dietary CEL, Matsuda index, and HOMA-IR, and these analyses were performed in smaller subsets (Supplementary Table 5.3). Furthermore, this association was also present when performing the original analysis with physical activity determined by questionnaires in this subset. As such, selection bias may have occurred.

When excluding those with previously known type 2 diabetes, all findings remained similar, except for associations between dietary AGEs and OGTT glucose iAUC. Specifically, higher intake of dietary CML and CEL, but not MG-H1, was associated with marginally higher OGTT glucose iAUC in the fully adjusted model, with β (SD) and (95% CI) of 0.05 (0.01,0.09), 0.04 (0.01,0.07), and 0.02 (-0.01,0.06), respectively (data not shown).

Sensitivity analyses in which we substituted income for educational level and BMI for waist circumference did not materially change the results. Finally, multinomial logistic regression analysis with tertiles of the Matsuda index and beta-cell function indices provided materially similar findings.

Discussion

In this population-based cohort, we observed no association between habitual intake of dietary AGEs CML, CEL, and MG-H1 and insulin sensitivity, fasting or post-OGTT glucose, or presence of prediabetes or type 2 diabetes. In contrast, higher intake of MG-H1 was associated with better beta-cell glucose sensitivity, but not with other indices of beta-cell function.

We found no association between habitual intake of dietary AGEs and insulin sensitivity, expressed as either the OGTT-derived Matsuda index or the HOMA-IR index. The effect of a low or high AGE diet on insulin sensitivity was hitherto only investigated in several smaller experimental studies, and meta-analyses suggest that a diet low in AGEs may improve insulin sensitivity[34]. However, due to methodological and practical limitations, including the usage of an outdated dietary AGE database, the non-gold standard HOMA-IR index to determine insulin sensitivity, and largely different cooking methods to modulate dietary AGEs, those studies do not justify the recommendation to restrict dietary AGE intake as a treatment for insulin resistance, beta-cell dysfunction, and ultimately type 2 diabetes. These limitations were addressed in two recent RCTs, of which results are mixed. In 2016, de Courten et al. showed an improvement in insulin sensitivity after a 2-week low compared to high AGE diet, based on cooking methods, in 20 abdominally obese individuals in a cross-over design[18]. In contrast, we recently reported no effect of a specifically designed 4-week diet low or high in AGEs on insulin sensitivity in 72 similar individuals in a carefully controlled intervention study in a parallel group design[17], which avoids many methodological drawbacks of the cross-over design, especially issues of wash-out and carry-over. Although the present observational study is not directly comparable to these two intervention studies, the combined findings of the present study with our recent intervention study[17] suggests that exposure to dietary AGEs is not associated with insulin resistance. Importantly, the intake of the concentration of AGEs in the lowest (quartile 1) and highest (quartile 4) consumers of the current cohort are in line with the intake achieved during low and high AGE diets of both our intervention study and the study by de Courten et al[18]. As such, the variation in AGE intake in the present observational study should have been sufficient to detect an association with insulin sensitivity and other clinical endpoints if such an association were present.

While we observed no association between dietary AGEs and the C-peptidogenic index and beta-cell rate sensitivity, both markers of early-phase insulin release, there was an association between higher intake of AGEs, especially MG-H1, and better beta-cell glucose sensitivity. This mathematically modelled index reflects the dependence of insulin secretion on absolute glucose concentration at any time point during the OGTT[29]. The seemingly beneficial association with greater intake of MG-H1, the most abundant dietary AGE, is surprising when compared to previous work suggesting either harmful effects of dietary AGEs on beta-cell function of mice[12], or no effects at all in humans[17, 18]. Specifically, a baked chow diet high in AGEs was shown to impair in insulin secretion in mice[12]. However, baked chow diets high in AGEs translate poorly to a human diet high in AGEs. In humans, a 2 or 4-week diet low or high in AGEs did not lead to changes in beta-cell function indices during an

intravenous glucose tolerance test (IVGTT)[18] or a hyperglycemic clamp[17], nor in any of the potential underlying mechanisms (inflammation, endothelial dysfunction, or lipid profile)[17]. Additionally, the association between MG-H1 intake and beta-cell glucose sensitivity in the present analyses did not translate into an association with lower fasting glucose, 2-hours post OGTT glucose, or OGTT glucose iAUC, or decreased odds of prediabetes of type 2 diabetes. On the one hand, this may provide insight on the potential limited biological relevance of this association, but on the other hand, this may also result from the cross-sectional design of this study.

We also observed inconsistent and opposing associations between dietary AGEs and HbA1c. After full adjustment for potential confounders, higher intake of MG-H1 was associated with marginally higher HbA1c. In contrast, among those with highest intake of CEL (Q4), HbA1c was lower, and this association was supported by a negative linear trend for HbA1c between quartiles of CEL intake. These opposing associations are difficult to explain and might reflect a chance finding. Also in light of the small effect sizes, their clinical relevance is small and likely irrelevant. Similarly, we observed an association between higher intake of CML and CEL with marginally higher OGTT glucose iAUC after exclusion of those with previously known type 2 diabetes. In The Maastricht Study, participants with type 2 diabetes have been oversampled by design, and its study population does not directly reflect the general population. However, as only the association with OGTT glucose iAUC was altered in this sensitivity analysis, it is unlikely that the oversampling of participants with type 2 diabetes has influenced our main results. Interestingly, we did observe an association between higher intake of CEL and lower Matsuda index in individuals with impaired kidney function. As the kidneys are responsible for clearance of plasma AGEs[33], those with impaired kidney function may be prone to prolonged exposure to dietary AGEs and their potential biological effects. However, in the current study, only 111 participants had impaired kidney function, and this impairment was only limited (median eGFR 55 ml/min/1.73m^2). Additionally, similar interactions were not found for associations between dietary AGEs and the HOMA-IR index. As such, these findings should be further explored in a more suited cohort with more individuals with impaired kidney function.

The present study has several strengths. Primarily, we are the first to investigate the association between intake of AGEs from the habitual diet and glucose metabolism in a population-based cohort. Next, because we determined AGE intake from the habitual diet, our results are a better indication of the effects of dietary AGEs as when dietary AGEs are increased by frying of food as opposed to boiling. Additionally, the deep phenotyping approach of The Maastricht Study allows for extensive adjustment for potential confounders. Particularly, we were able to investigate isolated beta-cell

function as we adjusted our analyses for insulin sensitivity. Finally, we determined beta-cell function not only as the more classical C-peptidogenic index, but also as mathematically modelled indices that may provide a better understanding of the insulin secretory response[29].

The present study also has several limitations. Mainly, detailed information on food preparation methods, such as cooking duration, are difficult to assess accurately using a FFQ and our dietary AGE database. Although the combination of an FFQ and our dietary AGE database is the best method to determine habitual AGE intake in a large cohort, this may have introduced inaccuracies in our estimates. Additionally, FFQs are prone to recall and information bias[35], however, we believe these effects are limited as sensitivity analyses among individuals with newly diagnosed type 2 diabetes revealed largely similar associations. Next, to infer causality between long-term habitual intake of AGEs and development of prediabetes and type 2 diabetes, prospective, preferably large, cohort studies with repeated measurements are required. Finally, we measured insulin sensitivity and beta-cell function with an OGTT, which is not the gold standard method. However, performing euglycemic and hyperglycemic clamps in this large population was not feasible. Additionally, the OGTT provides a more physiological stimulus of insulin secretion than a clamp or IVGTT.

To conclude, we found no association between habitual intake of dietary AGEs, insulin sensitivity, and presence of prediabetes or type 2 diabetes. Habitual intake of dietary MG-H1 was associated with higher beta-cell glucose sensitivity. Whether higher intake of dietary AGEs translates to increased incidence of prediabetes or type 2 diabetes on the long term should be investigated in a large prospective cohort study. Current evidence does not support the restriction of dietary AGEs to prevent prediabetes or type 2 diabetes.

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Supplementary Methods

Collection and measurement of plasma glucose, insulin, and c-peptide

Plasma for the assessment of insulin and C-peptide levels was collected in ethylenediaminetetraacetic acid (EDTA) tubes, stored on ice, separated after centrifugation (3000x g for 15 min at 4 °C), and stored at-80 °C until the assays were performed. The time between collection and storage was <2 h. Insulin and C-peptide were measured in never-thawed plasma by use of a custom duplex array of MesoScale Discovery (MesoScale Discovery, Gaithersburg, MD, USA). In short, 96 well-plates, with capture antibodies against insulin and C-peptide patterned on distinct spots in the same well, were supplied by the manufacturer. Samples (10 µL/well), detection antibodies and read buffer for electrochemiluminescence were applied according to manufacturer's instruction and plates were read using a SECTOR® 2400 Imager. Detection ranges of the assay were 35–25,000 pg/mL for insulin and 70–50,000 pg/mL for C-peptide. Interassay coefficients of variation for insulin and C-peptide were 10.1% and 8.2%, respectively. Insulin and C-peptide values were converted from pg/mL to pmol/L using a molar mass of 5808 g for insulin and 3010 g for C-peptide.

Plasma for the assessment of glucose was collected in sodium fluoride/potassium oxalate (NaF/KOx) tubes on ice. Fasting and 120-min-postload plasma glucose were measured in fresh samples with the enzymatic hexokinase method by use of two automatic analysers (i.e., the Beckman Synchron LX20 (Beckman Coulter Inc., Brea, CA, USA) for samples obtained between November 2010 and April 2012, and the Roche Cobas 8000 (Roche Diagnostics, Mannheim, Germany) for samples obtained thereafter). Plasma for the assessment of glucose at other time points during the OGTT was separated after centrifugation (3000x g for 15 min at 4 °C) and stored within 2 h at-80 °C until the assays were performed. Glucose was measured in these never-thawed samples with the enzymatic hexokinase method by use of the Roche Cobas 6000 (Roche Diagnostics, Mannheim, Germany). The Pearson correlation coefficient between fresh and frozen samples were 0.96 and 0.99, respectively, for fasting and 120-min-postload plasma glucose samples (n = 486 samples) in a quality control.

Supplementary Tables

Supplementary Table 5.1 Comparison of participants of the Maastricht Study with information on all potential confounders versus those with missing information on potential confounders.

Characteristic	**Included** (*n* = 6275)	**Excluded** (*n* = 1414)	*p*
Demographics			
Age (years)	59.8 ± 9.2	59.8 ± 8.6	0.891
Sex (% male)	49.9	52.5	0.082
Lifestyle			
Smoking – Never/Former/Current (%)	37.9/50.2/11.9	32.4/43.3/19.9	<0.001
Waist circumference (cm)			
Males	100.7 ± 11.9	102.2 ± 12.4	0.003
Females	89.2 ± 12.6	91.3 ± 14.1	<0.001
BMI (kg/m^2)	26.9 ± 4.4	27.6 ± 4.9	<0.001
Physical activity (h/week)	13.9 ± 8.2	13.2 ± 8.3	0.043
Education – Low/Medium/High (%)	33.4/27.9/38.7	41.2/26.2/32.6	<0.001
Biological			
Office Systolic blood pressure (mmHg)	133.6 ± 17.9	134.7 ± 18.7	0.043
Office Diastolic blood pressure (mmHg)	76.6 ± 9.8	75.5 ± 9.9	0.726
LDL cholesterol (mmol/L)	3.1 ± 1.0	3.0 ± 1.0	0.031
Total-to-HDL cholesterol ratio	3.6 ±1.2	3.7 ± 1.3	0.003
Triglycerides (mmol/L)	1.4 ± 0.8	1.5 ± 1.1	0.003
eGFR (mL/min/1.73 m^2)	82.0 ± 14.0	83.6 ± 15.3	<0.001
Medical history of CVD (% yes)	21.5	16.2	<0.001
Medication use			
Glucose-lowering medication (%yes)	16.8	28.4	<0.001
Anti-hypertensive medication (%yes)	43.3	37.0	<0.001
Lipid-lowering medication (%yes)	31.3	38.1	<0.001
Dietary intake			
Energy intake (kJ/day)	2139 ± 594	2161 ± 652	0.308
Alcohol intake (g/day)	8.2 [1.6,18.3]	6.3 [0.8,17.3]	0.004
Dutch Healthy Diet Index[*]	76.6 ± 14.5	74.7 ± 14.7	<0.001
Dietary CML (mg/day)	3.6 ± 1.3	3.6 ± 1.4	0.104
Dietary CEL (mg/day)	3.2 ± 1.5	3.1 ± 1.6	0.026
Dietary MG-H1 (mg/day)	24.9 ± 8.9	24.4 ± 9.1	0.111
Glucose metabolism			
Glucose metabolism status – NGM/Prediabetes/TD2M/Other (%)	61.8/15.1/23.2/0	51.5/13.9/31.1/3.5	<0.001
Fasting glucose (mmol/L) [†]	5.8 ± 0.8	6.3 ± 2.0	<0.001
HbA1c (%)[‡]	5.7 ± 0.8	6.0 ± 1.1	<0.001
2-hours post OGTT glucose [§] (mmol/L)	7.5 ± 4.0	8.0 ± 4.4	<0.001
OGTT glucose iAUC [‖] (mmol/L·min)	348 ± 281	367 ± 282	0.142
HOMA-IR index [¶]	1.4 [1.0,2.1]	1.5 [1.0,2.2]	0.001
Matsuda index [#]	3.52 [2.05,5.32]	3.31 [1.98,5.20]	0.085
C-peptidogenic index [**]	465 ± 1165	447 ± 683	0.736
Glucose sensitivity [††] (pmol min^{-1} m^{-2} mmol·L^{-1})	27.8 ± 18.4	24.9 ± 17.4	0.001
Potentiation factor [††]	1.6 ± 0.7	1.6 ± 0.6	0.234
Rate sensitivity[9] (pmol m^{-2} mmol·L^{-1})	251 ± 334	231 ± 263	0.190

Data are presented as means ± SD, medians [interquartile range], or percentages. Differences in characteristics for those included and excluded were tested with a one-way ANOVA and Kruskal-Wallis test for normally and non-normally divided variables, and with a chi-squared test for proportions. Reasons for missing data are provided in Figure 5.1.

[*]Modified version of the Dutch Healthy diet index that does not include alcohol intake. [†]Fasting glucose available in *n* = 6274. [‡]HbA1c available in *n* = 6268. [§]2-hours post OGTT glucose available in *n* = 5973. [‖]OGTT glucose iAUC available in *n* = 2700. [¶]HOMA-IR index available in *n* = 2608. [#]Matsuda index available in *n* = 2387. [**]C-peptidogenic index available in *n* = 2393. [††]Mathematically-modelled indices of beta-cell function available in *n* = 2440.

Abbreviations: CEL: N$^{\varepsilon}$-(1-carboxyethyl)lysine. CML: N$^{\varepsilon}$-(carboxymethyl)lysine. eGFR: estimated glomerular filtration rate. iAUC: incremental area under the curve. MG-H1: N$^{\delta}$-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. NGM: normal glucose metabolism. OGTT: oral glucose tolerance test. T2DM: type 2 diabetes mellitus.

Supplementary Table 5.2 Associations between dietary AGEs and the Matuda index in adults of The Maastricht Study stratified by kidney function below or above 60 ml/min/1.73m²

Dietary AGE (SD/day)	**eGFR < 60 ml/min/1.73m²** *n* = 111	**eGFR > 60 ml/min/1.73m²** *n* = 2276	**$P_{interaction}$**
CML			
Semi-adjusted β (95% CI)	-0.09 (-0.27,0.09)	0.01 (-0.04,0.05)	
Fully-adjusted β (95% CI)	-0.16 (-0.42,0.11)	-0.01 (-0.07,0.04)	0.005
CEL			
Semi-adjusted β (95% CI)	**-0.16 (-0.31,-0.01)**	-0.02 (-0.06,0.02)	
Fully-adjusted β (95% CI)	**-0.19 (-0.37,-0.02)**	-0.02 (-0.06,0.02)	0.003
MG-H1			
Semi-adjusted β (95% CI)	-0.09 (-0.27,0.09)	0.03 (-0.02,0.07)	
Fully-adjusted β (95% CI)	-0.13 (-0.37,0.11)	-0.02 (-0.07,0.03)	0.002

Multivariate-adjusted associations of dietary AGEs (in SD/day) and the Matsuda index tested with multiple linear regression analysis. Beta's and their (95% CIs) indicate the difference in standardized log-transformed Matsuda index per 1 SD difference in dietary AGE intake. Statistically significant findings are shown bold.

Semi-adjusted model 1: Adjusted for age and sex.

Fully-adjusted model 2: Additionally adjusted for cardiovascular risk factors (prior CVD, waist circumference, office systolic blood pressure, triglycerides, LDL-cholesterol, total/high-density lipoprotein ratio, use of lipid-lowering medication or antihypertensive medication, smoking habits, and kidney function) and lifestyle factors (energy intake, alcohol intake, the Dutch Healthy Diet index, physical activity, and educational level).

Supplementary Table 5.3 Associations between dietary AGEs and the Matuda and HOMA-IR index in adults of The Maastricht Study with available accelerometer data

Dietary AGE (SD/day)	**HOMA-IR index** B (95% CI)	**Matsuda index** B (95% CI)
CML		
Original analyses*	0.04 (-0.01,0.09)	-0.02 (-0.07,0.04)
Original analyses in smaller subset†	**0.06 (0.01,0.11)**	-0.05 (-0.11,0.02)
Sensitivity analyses in subset with additional correction for accelerometer data‡	**0.06 (0.01,0.11)**	-0.05 (-0.11,0.02)
CEL		
Original analyses*	0.02 [-0.01,0.06)	-0.03 (-0.07,0.01)
Original analyses in smaller subset†	0.03 (-0.01,0.07)	**-0.05 (-0.10,-0.00)**
Sensitivity analyses in subset with additional correction for accelerometer data‡	0.03 (-0.01,0.07)	**-0.05(-0.10,-0.00)**

Multivariate-adjusted associations of dietary AGEs (in SD/day) and the HOMA-IR index and Matsuda index tested with multiple linear regression analysis. Beta's and their (95% CIs) indicate the difference in standardized log-transformed HOMA-IR and Matsuda index per 1 SD difference in dietary AGE intake. Statistically significant findings are shown bold.

* Original fully-adjusted model, *n* = 2387 for HOMA-IR and *n* = 2387 for Matsuda.

† Original fully-adjusted model performed in the smaller subset of participants with available accelerometer data, *n* = 2026 for HOMA-IR and *n* = 1860 for Matsuda

‡ Additionally adjusted for cardiovascular risk factors (prior CVD, waist circumference, office systolic blood pressure, triglycerides, LDL-cholesterol, total/high-density lipoprotein ratio, use of lipid-lowering medication or antihypertensive medication, smoking habits, and kidney function) and lifestyle factors (energy intake, alcohol intake, the Dutch Healthy Diet index, physical activity, and educational level).

dietary AGEs in BLACK PUDDING WITH APPLES

CML: 4.8; CEL 7.7; MG-H1: 63.0 mg/100g

Chapter 6

A 4-week high-AGE diet does not impair glucose metabolism and vascular function in obese individuals

Armand M.A. Linkens, Alfons J.H.M. Houben, Petra M. Niessen, Nicole E.G. Wijckmans, Erica E.C. de Goei, Mathias D.G. van den Eynde, Jean L.J.M. Scheijen, Marjo P.H. van den Waarenburg, Andrea Mari, Tos T.J.M. Berendschot, Lukas Streese, Henner Hanssen, Martien C.J.M. van Dongen, Christel C.J.A.W. van Gool, Coen D.A. Stehouwer, Simone J.M.P. Eussen, Casper G. Schalkwijk.

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Abstract

Background: Accumulation of advanced glycation endproducts (AGEs) may contribute to the pathophysiology of type 2 diabetes and its vascular complications. AGEs are widely present in food.

Objective: We investigated whether restricting AGE intake improves risk factors for type 2 diabetes and vascular dysfunction.

Methods: Abdominally obese but otherwise healthy individuals were randomly assigned to a specifically designed 4-week diet low or high in AGEs in a double blind parallel-design. Insulin sensitivity, secretion, and clearance were assessed by a combined hyperinsulinemic-euglycemic and hyperglycemic clamp. Micro- and macrovascular function, inflammation, and lipid profile were assessed by state-of-art in vivo measurements and biomarkers. Specific urinary and plasma AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) were assessed by mass spectrometry.

Results: In 73 individuals (22 males, mean ± SD age and BMI 52 y ± 14, 30.6 kg/m2 ± 4.0), intake of CML, CEL, and MG-H1 differed 2.7, 5.3, and 3.7-fold between the low and high AGE diets, which led to corresponding changes of these AGEs in urine and plasma. Despite this, there was no difference in insulin sensitivity, secretion, or clearance, micro- and macrovascular function, overall inflammation, or lipid profile between the low and high dietary AGE groups (all *p* for treatment effects > 0.05).

Conclusions: This comprehensive RCT demonstrates very limited biological consequences of a 4-week diet low or high in AGEs in abdominally obese individuals.

Introduction

In vivo accumulation of advanced glycation endproducts (AGEs), a heterogeneous group of sugar-modified amino groups within proteins and other macromolecules, may drive the pathophysiology of type 2 diabetes[1-6] and its associated vascular dysfunction[7-11]. In addition to the endogenous formation of AGEs, AGEs also form in foods, especially those rich in sugar and protein or fat, when exposed to heat[12]. Since heating of food is widely employed due to favorable effects on sterility, flavor, and color, diets consumed in westernized societies significantly contribute to the body's exposure to AGEs[13]. Studies in animals[14, 15] and humans[13] suggest that these dietary AGEs are absorbed, and that a diet high in AGEs may induce endothelial dysfunction and inflammation[16, 17], insulin resistance[18], and beta-cell dysfunction[2]. However, whether reducing AGE intake improves risk factors for type 2 diabetes and vascular dysfunction remains controversial[19].

Studies so far mainly employed different cooking techniques to modulate dietary AGEs, such as boiling vs. frying, or did not match intervention and control diets for calories and macronutrients[20-24]. Additionally, dietary AGEs were almost exclusively estimated using an immunohistochemistry-based database[25] and not with the current gold standard instrumental method UPLC-MS/MS[26-28]. As such, observations from the above-mentioned studies[2, 16-18] may be attributed to other factors than dietary AGEs. Limitations from these previous studies were partly circumvented in a recent crossover randomized controlled trial (RCT) in obese individuals, where insulin sensitivity improved after a 2-week low compared to a high AGE diet[29]. However, several important knowledge gaps remain. Although dietary AGEs did not influence insulin secretion in response to an intravenous glucose bolus, this was not determined by c-peptide deconvolution and was not adjusted for the observed change in insulin sensitivity. Additionally, insulin clearance, increasingly recognized as an independent risk factor for type 2 diabetes[30], was not investigated in this study. Furthermore, micro- and macrovascular function, important contributors to insulin sensitivity[31], beta-cell function[32, 33], and cardiovascular risk[34-36], have not yet been investigated in a well-controlled RCT.

As such, the aim of the present study was to investigate the effects of dietary AGEs on factors involved in the pathogenesis of type 2 diabetes and on vascular function. To this end, we investigated in a double-blind parallel-design randomized controlled trial the effects of 4-week isocaloric and macronutrient-matched diet either low or high in AGEs on insulin sensitivity, secretion, and clearance, micro- and macrovascular function, inflammatory markers, and lipid profile in abdominally obese individuals using state-of-art methods.

Methods

Study design

In this double blind, parallel design, randomized controlled trial (deAGEing trial) participants were assigned at a 1:1 ratio to a 4-week dietary intervention low or high in AGEs. Randomization was performed remote from the recruitment center by an independent investigator to the research team and participants after email verification of correctness of the randomization criteria. The randomization sequence was generated by this independent researcher with an online randomization tool (randomization.com, original generator) using randomly permutated block sizes of 4, with stratification for age (below and above 50 years of age) and sex. Both the investigators and participants were formally blinded to the treatment allocation, and participants were instructed not to inform the investigators about the food items in their dietary intervention. The study dietician was aware of the treatment allocation.

Study Population

82 abdominally obese but otherwise healthy individuals were recruited by advertisements and enrolled at the Maastricht University Medical Center in Maastricht, The Netherlands, from November 2018 to March 2021. Eligible for inclusion were all individuals aged 18 and up and abdominally obese (waist circumference > 88 cm for females, > 102 for males[37]). Non-eligible were individuals with diabetes (fasting plasma glucose > 7.0 mmol/L, HbA1c > 6.5%, or self-reported use of glucose-lowering medication), CVD (medical history), current or previous smoking (cessation < 1 year ago), hyperlipidaemia (total cholesterol > 8 mmol/L, triglycerides > 4 mmol/L, or use of lipid-lowering medication), use of medication known to influence glucose metabolism or vascular function (e.g. glucocorticosteroids, NSAIDs), inability to stop anti-hypertensive medication for 8 weeks, pregnancy, unstable body weight (change > 3 kg in the last two months), use of dietary supplements within the previous month, or significant food allergies or intolerance.

Sample size calculation

The primary objective was to determine a change in insulin sensitivity assessed by the hyperinsulinemic-euglycemic clamp. A previous cross-over design RCT with a comparable study population and intervention diet found an improvement of 1.3 ± 1.8 mg/kg/min in insulin sensitivity after a 2-week low AGE diet relative to baseline[29]. Due to our parallel design, we expected greater variance between participants. However, our intervention period was four weeks rather than two, so the effect size was expected to be larger. As such, we expected an improvement of insulin sensitivity

of 1.5 ± 2.1 mg/kg/min. Using the PS Power and Sample Size Calculations Software program (version 3.1.2, 2014 by William D. Dupont and Walton D. Plumme), based on a two-tailed significance level of 0.05 and a power of 0.85, 36 individuals per group were needed to detect a statistical difference. Considering a drop-out rate of 12%, we included 41 participants per group, resulting in a total of 82 participants.

Run-in diet

Prior to the baseline measurement, all participants followed an isocaloric two-day run-in dietary schedule. Participants' habitual energy intake was assessed by a three-day food diary including two week days and one weekend day. The run-in diet contained an average amount of dietary AGEs, based on intake in a large population-based cohort 38, and was designed to exclude any influences of high AGE products consumed the days prior to the baseline measurement. Habitual intake of AGEs was assessed by a validated food frequency questionnaire (FFQ)[39] as described in detail in the Supplementary methods.

Dietary intervention

Intervention diets were constructed by a trained dietician and were energy- and macronutrient-matched. Both intervention diets adhered to the Dutch dietary guidelines for macro- and micronutrient intake[40] and contained 15% protein, 35% fat, 48% carbohydrates, and 2% fibers. With the use of our gold standard UPLC-MS/MS dietary AGE database that contains approximately 250 food items[28], a theoretical difference of approximately 75% in dietary AGEs was attained between diets. Participants prepared their food at home using predefined recurring weekly menus. Most food items were provided to the participants free of charge by means of a delivery service. Participants were instructed not to change their habitual portion sizes or habitual clock times of food intake, not to attempt changes in body weight, and not to consume food supplements during the duration of the study.

Compliance

Adherence to the dietary intervention was measured in three ways. First, participants kept a five-day dietary record in the first and last week of the intervention period. Second, participants were additionally contacted in the second and third week of the intervention period to assess food intake by a 24-hour dietary recall as described elsewhere[41]. Nutrient intake from these dietary records and recalls were determined using a nutrient software program (Compl-eat, Human Nutrition Wageningen University, Wageningen, The Netherlands). Third, free AGEs in 24-hour urine samples and plasma were compared between groups after the intervention.

Experimental visits

All measurements were performed by the head investigator. Measurements were conducted in a temperature-controlled room (T = 24 ± 0.5°C) after a 12-hour overnight fast and a 30-minute acclimatization period. Participants were instructed to refrain from alcohol and strenuous physical exercise for a period of 48-hours prior to each study day. Prior to the microvascular measurements, two venous catheters were fitted: one for sample collection, and the other for delivery of venous infusion. The infusion cannula was fitted in an antecubital vein of the left arm. The sampling cannula was placed in a suitable wrist vein of the ipsilateral hand, if possible, or an antecubital vein of the right arm.

Hyperinsulinemic euglycemic clamp

Insulin sensitivity was assessed by a 1 mU/kg/min euglycemic insulin clamp as described previously[42]. Briefly, insulin (NovoRapid, Novo Nordisk) was infused in a primed continuous manner for 120 minutes. Meanwhile, isoglycemia was maintained with a variable rate 20% glucose infusion. Metabolic insulin sensitivity was estimated from the steady-state glucose infusion rate (90–120 minutes of the clamp). Plasma glucose concentrations were measured in centrifuged venous blood samples (13,000 g for 30 seconds) with an on-site YSI2300 glucose analyzer (YSI, Yellow Springs, USA).

Insulin clearance was determined in the fasting state and during the steady-state period from the hyperinsulinemic clamp. Fasting insulin clearance was calculated as the ratio between fasting insulin secretion, determined by C-peptide deconvolution[43], and fasting plasma insulin concentration. Insulin clearance during the steady-state period of the hyperinsulinemic clamp was calculated as the ratio between insulin infusion rate and plasma insulin concentrations during the 90-120 minutes period. Plasma insulin concentrations were adjusted for endogenously secreted insulin, which was determined with the following formula:

$$\text{Endogenously-secreted insulin} = \text{insulin}_{\text{fasting}} * (\text{C-peptide}_{\text{90-120}} / \text{C-peptide}_{\text{fasting}})$$

This formula assumes that the endogenously-secreted insulin during the 90-120 minutes period of the hyperinsulinemic clamp changes in proportion to the c-peptide change in this period.

C-peptide suppression, reflecting insulin's potential to inhibit its own secretion[44], was calculated as percentage-change in average c-peptide concentration during the 90-120 minutes period relative to fasting values, using the following formula:

$$\text{C-peptide suppression} = ((\text{C-peptide}_{\text{90-120}} - \text{C-peptide}_{\text{fasting}}) / \text{C-peptide}_{\text{fasting}}) * 100\%$$

Hyperglycemic clamp

Beta-cell function was determined by a hyperglycemic clamp as described previously[45]. After the hyperinsulinemic euglycemic clamp, insulin infusion was discontinued and the glucose infusion rate was gradually decreased over a period of 60 minutes while maintaining fasting glucose levels. Next, a 30-minute square-wave step of hyperglycemia was applied to assess beta-cell function. This was achieved by a priming glucose dose (2.8 mmol/L above baseline), administered over 1 minute, followed by a variable 20% glucose infusion to maintain plasma glucose concentrations at the desired plateau. Venous blood samples for determination of plasma glucose, insulin, and c-peptide were obtained every 2 minutes for the first 10 minutes and every 5 minutes for the remainder of the step.

Insulin secretion rates (ISR) were calculated by means of C-peptide deconvolution[43]. The first-phase ISR response to the intravenous glucose bolus was expressed as the mean ISR incremental area under the curve (AUC) during the first 8 minutes after the glucose bolus. The second phase ISR response to the hyperglycemic clamp was expressed as the mean ISR AUC during 10-30 minutes. Because plasma glucose reached steady state mostly during the end of the hyperglycemic clamp, the second phase ISR response was also expressed as the mean ISR AUC during the last 5 minutes. Finally, beta-cell glucose sensitivity was expressed as the ratio between the ISR increment from baseline to 25-30 minutes and the corresponding glucose increment.

Contrast enhanced ultrasound

Insulin-mediated microvascular recruitment (IMMR) in forearm skeletal muscle during acute hyperinsulinemia was measured as described previously[42]. In short, microvascular blood volume of forearm skeletal muscle was measured with a Toshiba Aplio XG ultrasound system (Toshiba, Otawara, Japan) during continuous infusion of sulfur hexafluoride gas-filled microbubbles (SonoVue, Bracco diagnostics, Amsterdam, the Netherlands) in the fasting state and after 100 minutes of hyperinsulinemia. After 3 minutes of microbubble infusion, a steady state microbubble concentration is achieved and five real-team replenishment curves of 30 seconds were obtained after microbubble disruption by a high mechanical index ultrasound pulse. These replenishment curves were stored and analyzed offline in a blinded fashion using the CHI-Q software (Toshiba, Otawara, Japan). The replenishment curves were fitted to the exponential function $\gamma = A(1 - e^{-\beta t})$ and averaged as described elsewhere[46]. IMMR was expressed as the relative increase in microvascular blood volume during hyperinsulinemia. Collection of other microvascular measurements, and macrovascular measurements, is explained in detail in the Supplementary Methods.

Measurements in plasma

Soluble vascular cell adhesion molecule-1 (sVCAM-1), sICAM-1, high sensitivity C-reactive protein (CRP), tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), interleukin-8 (IL-8), adiponectin, serum amyloid A (SAA), insulin, and c-peptide were measured in EDTA plasma samples with commercially available 4-plex sandwich immunoassay kits (Meso Scale Discovery (MSD), Rockville, MD, US), as described elsewhere[47]. Soluble E-selectin (sE-selectin) was measured in EDTA plasma samples with sandwich ELISA (Diaclone, France). Von Willebrand factor (vWf) was determined in citrated plasma with sandwich ELISA (Dako, Glostrup, Denmark). Concentrations of vWf were expressed as a percentage of vWf detected in pooled citrated plasma of healthy volunteers. Gamma-GT, total cholesterol, HDL-cholesterol, and triglycerides were determined in serum using enzymatic and colorimetric methods by an automatic analyzer (Beckman Synchron LX20, Backman Coulter Inc., Brea, USA). LDL-cholesterol was determined via the Friedewald formula[48].

Advanced glycation endproducts, dicarbonyls, and oxidative stress markers in plasma and urine

Free and protein-bound AGEs in plasma and free AGEs in urine were analyzed as described in detail elsewhere[49]. In brief, for protein-bound and free AGEs in plasma, 25 µL and 50 µL of plasma was used, respectively. For free AGEs in urine, 40 µL of urine were used. All samples were derivatized with butanolic hydrochloric acid and subsequently detected in ESI positive multiple reaction monitoring (MRM) mode using a Xevo TQ MS (Waters, Milford, USA). Quantification of N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) was performed by calculating the peak area ratio of each unlabeled peak area to the corresponding internal standard peak area. In plasma, the intra- and inter-assay variation of protein-bound CML and CEL were between 4.8 and 9.7% and for free CML, CEL and MG-H1 between 2.8 and 7.1%. In urine, the intra- and inter-assay variation of free CML, CEL and MG-H1 were between 3.7 and 6.6%. Measurement of dicarbonyls in plasma was performed as described in detail elsewhere[50]. Measurement of AGE-accumulation in skin by skin autofluoresence is described in detail in the supplementary methods.

For the quantification of the urinary biomarkers CML, CEL, MG-H1, pyrraline, MGO, 8-Oxo-2'-deoxyguanosine (8-Oxo-dG), and N^{2}-(1-carboxyethyl)-2'-deoxyguanosine (CEdG), 50 µL of urine was mixed with internal standard mix and derivatized with acidified o-phenylenediamine and subsequently separated on a UPLC C18-column using ion-pair solvents. All biomarkers were detected in positive multiple-

reaction-monitoring mode, with MGO as a quinoxaline adduct. All biomarkers were successfully separated and detected with UPLC-MS/MS with a run-to-run time of 14 minutes. Linearity of all markers was tested in water and urine matrix and showed good correlation ($r^2 > 0.99$) with an intra- and inter-assay coefficients of variations (CV, %) of ~5%.

Statistics

Analyses were conducted using a pre-specified analysis plan, blinded for randomization, using SPSS version 25 for Windows (IBM Corporation, Armonk, NY, USA). Data are presented as means ± SD, medians [interquartile range], or percentages, as appropriate. Outcomes were assessed in an intention-to-treat complete case analysis. Differences within groups after the intervention were assessed by a paired-samples t-test, whereas differences between groups after the intervention period were assessed by a one-factor ANCOVA with sex, age, and the baseline value of the outcome of interest as a covariate. p values of < 0.05 were considered statistically significant.

We investigated the robustness of our findings in sensitivity analyses. To adjust for differences in macronutrient content between intervention diets, we additionally adjusted the ANCOVA model for intake of carbohydrate, protein, and fat as energy-percentages. Secondly, we used multiple linear regression analysis to investigate whether indices of dietary AGE intake, being self-assessed AGE intake during the intervention, and free AGEs in plasma and urine, were also associated with outcomes. The fully adjusted regression model was adjusted for age, sex, and intake of carbohydrate, protein, and fat as energy-percentages.

Study approval

This study was approved by the Maastricht University Medical Center ethics committee, performed in accordance with the Declaration of Helsinki, and registered at both international and national trial registries (clinicaltrials.gov: NCT03866343, trialregister.nl: NTR7594). All participants provided written informed consent.

Results

Baseline characteristics

Of 82 enrolled participants, insulin sensitivity was available in 73 participants (Figure 6.1). Dropouts occurred unrelated to the dietary intervention, and characteristics of these participants were largely comparable to those included in the complete case analysis (Supplementary Table 6.1). By design, all participants were abdominally obese, non-smokers, and free of apparent cardiovascular disease (Table 6.1). We included more females (*n* = 51) than males (*n* = 22) by chance. Baseline characteristics were not different between groups, except for habitual intake of dietary CML and CEL, which was higher in the low AGE group compared to the high AGE group (age, sex, and energy-intake adjusted $p = 0.007$ for CML, $p = 0.004$ for CEL, and $p = 0.08$ for MG-H1). This was attributable to higher consumption of several high AGE foods by participants in the low AGE group (beef stew, peanuts and peanut butter, and chocolate milk).

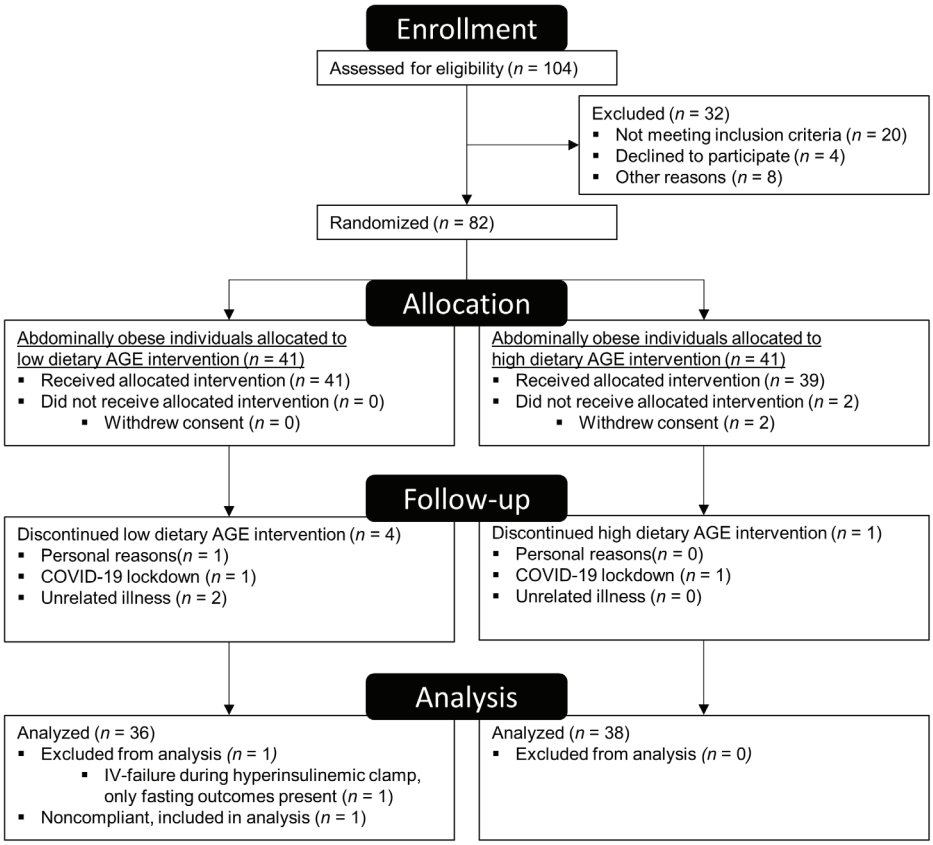

Figure 6.1 Consort flowchart

Dietary intake during the intervention

The intake of dietary AGEs CML, CEL, and MG-H1, assessed from two 5-day food diaries at week 1 and 4 of the dietary intervention, were markedly different during the low and high AGE diets, with daily intake (mg) for CML of 2.63 ± 0·68 mg/day vs. 7.02 ± 1.60 mg/day, for CEL of 1.69 ± 0.40 mg/day vs 9.03 ± 2.23 mg/day, and for MG-H1 of 13.44 ± 3.15 mg/day vs. 49.67 ± 13.92 mg/day (Table 6.2). This was also confirmed by 24-hour recalls in week 2 and 3 of the dietary intervention (Supplementary Table 6.2). Compared to habitual intake assessed by a food frequency questionnaire, all participants showed decreased intake in at least one of three dietary AGEs during the low AGE diet, and increased intake in at least one of three dietary AGEs during the high AGE diet (Supplementary Figure 6.1). We also determined the habitual intake of dicarbonyls, highly reactive compounds that give rise to rapid formation of AGEs. The intake of these dietary dicarbonyls were also different during both diets, although less marked (Table 6.2).

Energy intake during the intervention and body weight after the intervention were not statistically different between groups (Table 6.2 and Table 6.3). Although the intervention diets were designed to be macronutrient-matched, intake of energy as fat was slightly lower in favor of carbohydrates during the low AGE diet: mean energy % ± SD of 31.5 ± 2.6 En% vs. 35.8 ± 3.1 En%, $p < 0.001$ for fat, and 48.4 ± 2.6 En% vs. 44.5 ± 2.8 En%, $p < 0.001$ for carbohydrates (Supplementary Figure 6.2). Intake of fiber was marginally but statistically higher during the low AGE diet compared to the high AGE diet: 2.1 ± 0.2 En% vs. 2.3 ± 0.1 En%, $p = 0.001$. Consumption of alcohol was not statistically different between both groups (data not shown). There also were some differences in micronutrient intake (Supplementary Table 6.3). No diet-related adverse effects were reported.

Table 6.1 Characteristics of abdominally obese individuals that completed the low or high AGE diet at baseline

Characteristic	Low AGE (*n* = 35)	High AGE (*n* = 38)
Demographics		
Age (years)	51 ± 14	53 ± 14
Males/Females	10/25	12/26
Weight (kg)	87.6 ± 14.3	88.8 ± 13.4
Waist circumference (cm)		
Men	107 ± 5	108 ± 7
Women	101 ± 9	100 ± 8
BMI (kg/m^2)	30.4 ± 4.1	30.8 ± 4.0
24-hour systolic BP[1] (mmHg)	126 ± 13	124 ± 9
24-hour diastolic BP[1] (mmHg)	80 ± 9	77 ± 7
Biological		
Fasting glucose (mmol/L)	4.9 ± 0.4	5.1 ± 0.5
Fasting insulin[2] (pmol/L)	9.6 ± 2.9	10.7 ± 5.1
HbA1c (%)	5.3 ± 0.2	5.3 ± 0.4
HDL cholesterol (mmol/L)	1.4 ± 0.4	1.3 ± 0.3
LDL cholesterol (mmol/L)	3.4 ± 0.9	3.7 ± 0.8
Triglycerides (mmol/L)	1.2 ± 0.4	1.6 ± 0.8
Fatty liver index (unitless)	60 ± 21	63 ± 21
eGFR (ml/min/1.73m^2)	89.9 ± 14.9	88.4 ± 17.0
Habitual dietary intake[3]		
Energy intake (kcal/day)	2409 ± 756	2165 ± 862
Dutch Healthy Diet index	80.5 ± 17.6	83.7 ± 13.4
CML (mg/day)	4.59 ± 1.84	3.57 ± 1.44
CEL (mg/day)	4.43 ± 2.02	3.24 ± 1.26
MG-H1 (mg/day)	29.37 ± 10.45	24.76 ± 10.11
MGO (mg/day)	3.80 ± 1.58	3.67 ± 1.46
GO (mg/day)	3.75 ± 1.37	3.43 ±1.51
3-DG (mg/day)	15.02 [11.95,27.44]	14.04 [9.97,23.47]
Primary outcomes		
Insulin sensitivity (mg/kg/min)	4.5 ± 1.8	4.3 ± 2.0
First-phase insulin secretion[3] (pmol/min/m^2)	264 ± 161	265 ± 154
IMMR[2] (%)	9 ± 42	5 ± 32

Data are presented as means ± SD, medians [interquartile range], or percentages. Abbreviations: 3-DG: 3-Deoxyglucose. BP: blood pressure. CEL: N$^{\varepsilon}$-(1-carboxyethyl)lysine. CML: N$^{\varepsilon}$-(carboxymethyl)lysine. eGFR: estimated glomerular filtration rate. GO: Glyoxal. IMMR: Insulin-mediated microvascular recruitment. MGO: Methylglyoxal. MG-H1: N$^{\delta}$-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. [1] Low AGE *n* = 33, High AGE *n* = 37. [2] Low AGE *n* = 34, High AGE *n* = 38. [3] Low AGE *n* = 35, High AGE *n* = 36

Table 6.2 Average daily AGE-, dicarbonyl- and energy- intake during the intervention

Nutrient	Low AGE (*n* = 34)[1]	High AGE (*n* = 38)	Low vs High *p*
AGEs (mg/day)			
CML	2.63 ± 0.68	7.02 ± 1.60	<0.001
CEL	1.69 ± 0.40	9.03 ± 2.23	<0.001
MG-H1	13.44 ± 3.15	49.67 ± 13.92	<0.001
Dicarbonyls (mg/day)			
MGO	2.97 ± 0.91	3.82 ± 1.14	<0.001
GO	2.81 ± 0.72	3.28 ± 0.84	<0.001
3-DG	13.66 ± 5.23	19.45 ± 6.30	<0.001
Energy (kcal/day)			
Energy intake[2]	2004 ± 478	2138 ± 600	0.263

Daily intakes (means ± SD, medians [IQR]) were assessed from two five-day dietary logs in week 1 and week 4 of the intervention. Differences between intervention groups were tested by a one-factor ANCOVA with energy intake, sex, and age as covariates. [1]Dietary logs were not returned by one participant in the low AGE group. [2]Energy intake was not included as a covariate.

Table 6.3 Effects of a 4-week diet low and high in AGEs on main outcomes of interest in abdominally obese individuals.

Variable	Within-group change **Low AGE** mean [95% CI]	Within-group change **High AGE** mean [95% CI]	Overall difference **Low vs high** mean [95% CI]
Weight (kg)	**-0.7 [-1.3,-0.2]**	-0.2 [-0.6,0.3]	-0.5 [-1.3,0.2]
AGEs in urine[1]			
CML (nmol/mmol creatinin)	11.1 [-77.8,99.9]	121.5 [-9.9,252.8]	-127.2 [-280.7,26.3]
CEL (nmol/mmol creatinin)	-29.7 [-66.3,6.9]	292.2 [206.7,377.8]	**-300.9 [-387.8,-213.9]**
MG-H1 (nmol/mmol creatinin)	**-601.2 [-981.5,-220.9]**	2053 [1302,2805]	**-2607 [-3296,-1918]**
Pyrraline (nmol/mmol creatinin)	**233.2 [87.1,379.2]**	1151 [787.3,1514]	**-902.0 [-1297,-507.0]**
AGEs in plasma[1]			
Free CML (nmol/L)	-5.3 [-16.2,5.5]	8.8 [-0.2,17.8]	**-12.5 [-22.8,-2.2]**
Free CEL (nmol/L)	-3.6 [-7.5,0.3]	37.6 [27.3,47.9]	**-39.5 [-50.1,-28.9]**
Free MG-H1 (nmol/L)	-18.7 [-40.0,2.5]	115.7 [79.3,152.0]	**-133.2 [-170.0,-96,4]**
Glucose metabolism			
Insulin sensitivity (mg/kg/min)	0.05 [-0.54,0.65]	0.38 [-0.10,0.85]	-0.25 [-0.96,0.47]
1st phase ISR[1] (pmol/min/m^2)	21 [-3,44]	1 [-25,27]	17 [-18,51]
2nd phase ISR[3] (pmol/min/m^2)	8 [-8,24]	-2 [-15,11]	11 [-9,30]
2nd phase ISR steady state[3] (pmol/min/m^2)	9 [-14,31]	8 [-10,26]	1 [-26,28]
Steady-state insulin clearance[3] (L/min/m^2)	-0.01 [-0.04,0.01]	-0.01 [-0.03,0.02]	-0.01 [-0.04,0.02]
Micro and macrocirculation			
Flow-mediated dilation (%)	-0.0 [-0.7,0.7]	0.2 [-1.3,1.6]	-0.1 [-1.5,1.2]
IMMR[3] (%)	-1.2 [-20.6,18.1]	5.5 [-8.6,19.7]	-3.1 [-19.5,13.4]
Plasma biomarkers			
Endothelial dysfunction Z-score (SD)	0.06 [-0.13,0.25]	-0.06 [-0.23,0.11]	0.11 [-0.13,0.35]
Inflammatory markers Z-score (SD)	0.09 [-0.09,0.28]	0.09 [-0.28,0.10]	0.18 [-0.08,0.44]
Oxidative stress and DNA glycation			
8-Oxo-dG (nmol/mmol creatinin)	0.0 [-0.2,0.2]	-0.2 [-0.6,0.3]	0.0 [-0.4,0.4]
CeDG (nmol/mmol creatinin)	**-0.1 [-0.2,-0.0]**	0.2 [-0.0,0.3]	**-0.2 [-0.3,-0.1]**

Within-group changes were evaluated with a paired-samples t test. Overall differences after the low compared to high AGE diet were evaluated with a one-way ANCOVA with adjustment for age, sex, and the baseline variable of interest. Abbreviations: 8-Oxo-dG: 8-Oxo-2'-deoxyguanosine. CEDG: N_2-(1-carboxyethyl)-2'-deoxyguanosine CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. IMMR: Insulin-mediated microvascular recruitment. ISR: Insulin secretory response. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. *n* = 35 for the low AGE group and 38 for the high AGE group. [1] *n* = 34 for low AGE, *n* = 36 for high AGE. [2] *n* = 34 for low AGE, *n* = 38 for high AGE.

AGEs and dicarbonyls in plasma, urine, and skin

In line with their intake, levels of free AGEs CML, CEL, and MG-H1 in plasma and 24-hour urine were higher after the high AGE diet as compared to low AGE diet (Table 6.3, Supplementary Table 6.4, Figure 6.2), although the difference for free CML in urine did not reach statistical significance (p = 0.07). The AGE pyrraline, suggested to be derived mainly from food intake and not from endogenous formation[51], also showed increased levels in urine after the high compared to low AGE diet. Protein-bound AGEs in plasma were not statistically different after the low compared to high AGE diet (Figure 6.2). Skin autofluoresence, an estimate of fluorescent AGE accumulation in skin[52], was also not statistically different after the low compared to high AGE diet (Supplementary Table 6.3). Of note, one participant allocated to the low AGE diet showed a profound increase in urinary and plasma levels of free CEL and MG-H1 after the intervention diet (Figure 6.2, black dots). This participant

was deemed non-compliant and was not included in the analyses regarding AGEs in plasma and urine. In line with our intention-to-treat design, this participant was included in all other analyses. Exclusion of this participant did not materially change all other outcomes (data not shown).

Levels of AGEs in 24-hour urine correlated with their corresponding average daily intake during the intervention (Pearson's r = 0.26 (p = 0.03) for CML, r = 0.57 ($p < 0.001$ for CEL), and $r = 0.61$ ($p < 0.001$) for MG-H1). Although intake of dicarbonyls was also slightly higher during the high AGE diet, we observed no difference in their levels in plasma and 24-hour urine after both diets (Supplementary Table 6.3).

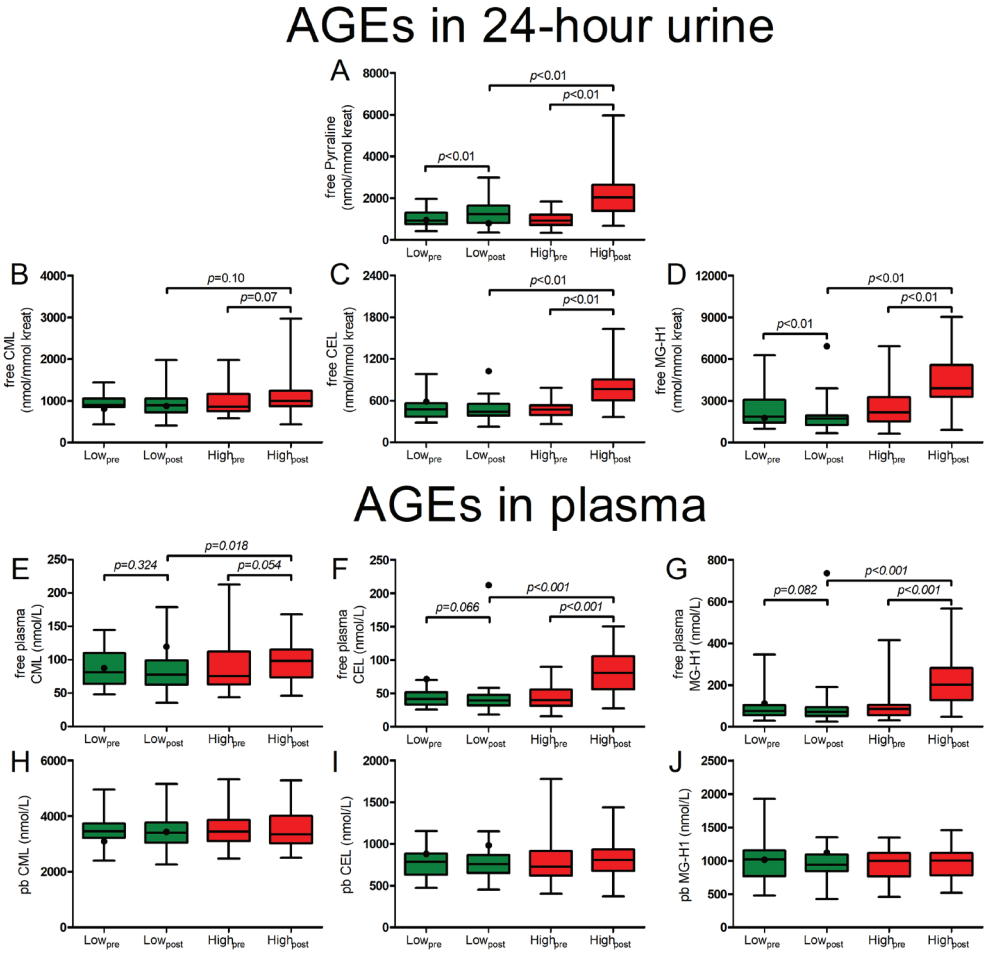

Figure 6.2 Free AGEs in 24-hour urine and plasma before and after a 4-week low or high AGE diet in abdominally obese individuals (n = 36 and n = 38 for low and high AGE diet). One participant was deemed noncompliant and was not included in statistical analyses of these variables. This participant is shown as a black dot.

Glucose metabolism

Fasting indices of glucose metabolism (plasma glucose, plasma insulin, plasma c-peptide, insulin secretion, and insulin clearance) were not statistically different after the low and high AGE diets (Supplementary Table 6.5).

Insulin sensitivity was not statistically different after the low compared to the high AGE diet, with overall difference of -0.5 mg/kg/min [-1.3,0.2], representing a ~10% non-significant change (Table 6.3). Additional adjustment for plasma insulin (M/I) did not materially change these findings (data not shown). Likewise, insulin clearance and c-peptide suppression (reflecting insulin's potential to inhibit its own secretion[44]) were not statistically different after both diets (Table 6.3 and Supplementary Table 6.5).

Finally, we determined insulin secretion indices during the hyperglycemic clamp. Following the intravenous glucose bolus, plasma glucose was sharply raised by the desired increment of 2.80 mmol/L (Supplementary Figure 6.3). The low or high AGE diet had no effect on the corresponding increase in first-phase insulin secretion rate (ISR), with an overall difference of 179 pmol/min/m^2 [-19,52] (Table 6.3). Adjusting for the variability in glucose increment by adding it as a variable in the ANCOVA model did not materially change these findings (data not shown). After the glucose bolus, plasma glucose was maintained at the 2.80 mmol/L increment by a variable glucose infusion. The resulting second-phase ISR during the remaining 20 minutes of the hyperglycemic clamp, as well as the last 10 minutes only (at which plasma glucose was more stable) were not statistically different after the low compared to high AGE diet (Table 6.3 and Supplementary Table 6.5). Beta-cell glucose sensitivity was also not different after the low compared to the high AGE diet (Supplementary Table 6.5). Additionally adjusting these indices for insulin sensitivity did not materially change these findings (data not shown).

Microvascular function

Insulin-mediated microvascular recruitment (IMMR) in skeletal muscle and the skin microvascular response to local heating, reflecting in vivo skeletal muscle and skin microvascular function, were not statistically different after the low compared to the high AGE diet: the overall difference for IMMR was -3.1 % [-19.5,13.4] (Table 6.3 and Supplementary Table 6.6). Likewise, microvascular retinal vessel calibers, skin microvascular flowmotion, the z-score of endothelial dysfunction plasma biomarkers, or the individual markers were also not statistically different between the low compared to the high AGE diet (Table 6.3, Supplementary Table 6.6).

Macrovascular function

Macrovascular endothelial function, assessed by flow-mediated dilation, was not statistically different after the low compared to the high AGE diet: the overall difference was -0.1 % [-1.5,1,2] (Table 6.3). Aortic stiffness, assessed by carotid-femoral pulse wave velocity (cfPWV), carotid stiffness, assessed by carotid distensibility coefficient (DC) and carotid Young's Elastic Modulus (YEM), carotid intima-media thickness (IMT), augmentation index, and 24-hour systolic and diastolic blood pressure were not statistically different following both diets (Supplementary Table 6.6). Additional adjustment of the macrovascular analyses for systolic blood pressure did not materially change the results (data not shown).

Inflammatory markers

With all the plasma inflammatory markers combined in a z-score, there was no statistically significant difference after the low compared to the high AGE diet, with overall difference of 0.18 SD [-0.08,0.44] (Table 6.3). Of the individual plasma inflammatory markers, only adiponectin decreased after the low AGE diet, which resulted in an overall difference of -1.54 ug/ml [-2.37,-0.71] after the low compared to the high AGE diet (Supplementary Table 6.7).

Absolute monocyte count was reduced after the high AGE diet, which resulted in overall difference of 0.05 10^9/L [0.01,0.08] after the low compared to the high AGE diet (Supplementary Table 6.7). Counts of leucocytes, segmented granulocytes, lymphocytes, eosinophils, and basophils were not statistically different after the low compared to the high AGE diet.

Cholesterol and liver markers

Triglycerides, LDL and HDL-cholesterol, Gamma-GT, and the fatty liver index were not statistically different after the low compared to the high AGE diet (Supplementary Table 6.8).

Markers of DNA-glycation

N^2-(1-carboxyethyl)-2'-deoxyguanosine (CEdG) levels in 24-hour urine, reflecting DNA glycation, decreased after the low AGE diet and increased after the high AGE diet, resulting in a statistically significant overall increase after the high compared to low AGE diet (Table 6.3). 8-Oxo-2'-deoxyguanosine (8-Oxo-dG) levels in 24-hour urine,

reflecting DNA oxidation, were not statistically different after the low compared to high AGE diet.

Sensitivity analyses

The increase in plasma adiponectin and urinary CEdG, and the decrease in absolute monocyte count after the low compared to high AGE diet remained statistically significant with further adjustment of the ANCOVA model for carbohydrate-, protein-, and fat intake (data not shown). To further assess the robustness of these findings, we also performed multiple linear regression to determine associations between indices of AGE intake and these outcomes. While adjusting for age, sex, and intake of carbohydrate-, protein-, and fat, daily intake of CML, CEL, and MG-H1 were positively associated with plasma adiponectin, while levels of free CML in urine were inversely associated with plasma adiponectin (Supplementary Table 6.9). There was a trend for an inverse association between free plasma CML and absolute monocyte count (p = 0.05), while intake of all AGEs and all free AGEs in plasma and urine were positively associated with urinary CeDG (Supplementary Table 6.9).

Interestingly, with additional adjustment for macronutrient intake in the ANCOVA model, some additional outcomes became statistically different after the low compared to high AGE diet. cfPWV and serum HDL were lower, while serum triglycerides were higher after the low compared to high AGE diet, with an overall difference of -0.78 m/s [-1.53,-0.02], -0.10 mmol/L [-0.19,-0.01], and 0.23 mmol/L [0.05,0.40], respectively (data not shown). However, only for serum HDL and triglycerides, and not for cfPWV, we found statistically significant associations with indices of dietary AGE intake. Intake of CML, CEL, and MG-H1 were positively associated with serum HDL (Supplementary Table 6.9). Intake of CML, CEL, and MG-H1, and free CEL and MG-H1 in plasma and urine were inversely associated with serum triglycerides (Supplementary Table 6.9).

Additionally adjusting for magnesium and selenium intake, micronutrients associated with insulin sensitivity, did not materially change our findings (data not shown). Of all other outcomes, only serum HDL become lower after the low compared to high AGE diet after additional adjustment for selenium intake (data not shown).

Discussion

Here, we present short-term biological effects of dietary AGEs using state-of-art methods. Despite a marked difference in intake of dietary AGEs, a 4-week diet low compared to high in AGEs did not change insulin sensitivity, secretion, and clearance, vascular function, and overall inflammation of healthy but abdominally obese individuals.

We supplied a profound difference in AGE intake between diets, being 167% for CML, 434% for CEL, and 270% for MG-H1, obtained without drastically altering food preparation methods and with similar energy content. Importantly, the comparison with habitual AGE intake revealed that indeed all participants lowered or increased their intake in line with their treatment allocation. Compliance was further confirmed by the increased levels of free AGEs in 24-hour urine and plasma after the high compared to low AGE diet. Despite this, we observed no difference in insulin sensitivity after both diets. In contrast, de Courten et al. showed an improvement in insulin sensitivity by 2.1 mg/kg/min after a 2 week low compared to high AGE diet in a well-controlled RCT with crossover design[29]. As AGE intake was estimated in both studies using the same UPLC-MS/MS dietary AGE database, they are directly comparable. Interestingly, AGE intake during our low AGE diet was lower, while it was also higher during our high AGE diet when compared to the corresponding diets by de Courten et al[29]. Other differences that may have contributed to our inconsistent findings are the crossover design, study population, and design of the dietary intervention. Although crossover bias was statistically excluded in their analyses, an incomplete washout could still have contributed to the change in insulin sensitivity observed after the second intervention period. Additionally, they included slightly younger and more insulin sensitive participants (age 34 ± 10 years, M-value 7.0 ± 2.5 mg/kg/min), and effects of low and high AGE diet might be more profound in this group when compared to our older and less insulin sensitive participants. Importantly, differences in AGE content of low and high AGE diets in the de Courten study were achieved by carefully matching low and high AGE food products based on differences in cooking techniques[53]. In contrast, our low and high AGE diet were designed as stand-alone diets, and differences in AGEs were not solely achieved by cooking methods. Although this may explain why we also found more differences in micronutrients between our diets, our approach reduces the possibility that large differences in food preparation methods have confounded our results. Furthermore, it is unlikely that the small difference in fat content between our low and high AGE diets masked a change in insulin sensitivity, as far greater differences were needed in intervention trials to elicit such an effect[54], and we also found no difference in insulin sensitivity while adjusting for macronutrient intake. Another possibility is that

an acute dietary AGE-induced change in insulin sensitivity is not sustained after 2 weeks. However, in line with the unchanged insulin sensitivity in the present study, all other outcomes of glucose metabolism also showed no change. The first-phase insulin secretion response, reduced in individuals with impaired glucose tolerance[55], was not changed after the low or high AGE diet. This was also reported by de Courten et al[29]. We extend on these findings by also investigating the second-phase insulin response, beta-cell glucose sensitivity, and fasting insulin secretion, which were also all unaffected by the low or high AGE diet. Importantly, our indices of beta-cell function were determined by the gold standard hyperglycemic clamp. Furthermore, these findings were independent of hepatic insulin clearance and insulin sensitivity, as we determined insulin secretion rates by c-peptide deconvolution[43], which is not affected by hepatic insulin clearance, and adjusted our final analyses for insulin sensitivity. Furthermore, we are the first to investigate the effects of dietary AGEs on insulin clearance, which is increasingly recognized as an important determinant of T2DM risk[30, 56] and was recently shown to predict T2DM in Native Americans, independent of insulin sensitivity and secretion[30]. However, we observed no difference in insulin clearance during the fasting state as well as during hyperinsulinemia after the low or high AGE diet.

Despite the increase of free AGEs in plasma, indicating higher exposure of the vascular endothelium to AGEs, we observed no deterioration of an extensive panel of macrovascular and microvascular function measurements. Ultimately, this is not surprising, as we did also not observe supporting differences in the potential underlying pathophysiological mechanisms (24-hour blood pressure, insulin sensitivity, lipid profile, inflammatory markers, plasma biomarkers of endothelial dysfunction, and oxidative stress). If anything, there was a slight improvement in inflammation and lipid profile after the high AGE diet, as apparent from the decrease in total monocyte count and serum triglycerides, and increase in adiponectin and serum HDL. The lower cfPWV observed after the low compared to high AGE diet occurred only when additionally adjusting for macronutrient intake and is likely a chance finding. In contrast to all other outcomes, we did not observe an association between any of the markers of dietary AGE intake and cfPWV. Overall, these findings are in line with those of our two previous observational studies in the population-based cohort of The Maastricht Study, where we observed no association between habitual intake of dietary AGEs and both arterial stiffness[38] and generalized microvascular function[57]. These results are seemingly in contrast with the role of endogenously-formed AGEs in microvascular dysfunction[58, 59] and arterial stiffening[60-62]. Specifically, AGEs have been linked to arterial stiffness and microvascular dysfunction in several studies[58, 59] via mechanisms that include collagen crosslinking within the vascular wall[62] and endothelial dysfunction via stimulation of the receptor for AGEs (RAGE)[63, 64]. However,

as collagen-crosslinking occurs during the formation of AGEs in the vascular wall, and dietary AGEs did not show affinity for RAGE[65], both mechanisms are unlikely to apply to AGEs of dietary origin. Combined, this suggests a limited role of dietary AGEs in micro and macrovascular function in humans.

Interestingly, we did observe increased levels of CEdG in urine after the high AGE diet, reflecting DNA glycation by MGO[66]. Formation of CEdG by MGO is considered highly mutagenic, as it is accompanied by guanine transversions and base deletions[67], and single-strand DNA breaks[68]. As such, the finding of increased CEdG after the high AGE diet provides an interesting topic of future research given the increased risk of several cancer types with higher habitual AGE intake[69-72].

Our broad array of null findings are not in agreement with those of previous animal and human studies. In mice, baked chow diets high in AGEs led to impaired insulin secretion[2], insulin resistance and T2DM[73], and arterial stiffness[74]. However, the usage of baked chow diets may have led to other effects than solely increasing dietary AGEs, such as decreased vitamin bioavailability and increased acrylamide formation. Additionally, AGE levels in baked chow may be higher than those in human food. As such, the relevance of these findings in mice for humans seems limited. Data in humans are less consistent, but meta-analyses of RCTs suggest that a high AGE diet leads to insulin resistance[75], inflammation and endothelial dysfunction[16], and atherogenic dyslipidemia[17]. However, most RCTs in these meta-analyses are deemed of low methodological quality[18, 19, 76]. Additionally, dietary AGEs were mostly modulated by employing largely different food preparation methods (i.e. steaming/boiling vs. grilling/frying). As such, the limitations of using baked diets described above also applies to these human studies. Furthermore, most intervention diets were not matched for energy content or energy intake was not reported[21, 22, 24]. As such, effects observed in these studies may not be attributed to dietary AGEs alone. Importantly, AGEs were measured in most studies using ELISA, which shows limited reliability compared to the gold standard UPLC/MS-MS. Thus, the true levels of AGEs in food, plasma, and urine in these studies are unknown. Another limitation was that in most previous trials both participants and investigators were not masked for treatment allocation[20-24, 77, 78]. Therefore, current data from literature is insufficient to conclude that dietary AGEs pose a threat to human health[19].

The present study has several strengths. Mainly, we measured an extensive panel of outcomes concerning glucose metabolism, vascular function, and inflammation using state-of-art methods. Additionally, AGEs and dicarbonyls in food, plasma, and urine were determined using the gold standard UPLC-MS/MS. Our intervention diets were specifically-designed and not solely based on food preparation methods,

making them directly translatable to daily practice. Compliance to the intervention diets was enhanced by frequent checkups by our trained dieticians and by delivering most food items to the participants free of charge. Compliance was confirmed by both food diaries, 24-hour recalls, and AGEs in plasma and urine. Furthermore, both the investigators and participants were blinded to the participant's treatment allocation, and the investigators remained blinded until the statistical analyses were finalized. Although it is theoretically possible that participants discovered their allocation themselves, this is not evident as also the low AGE diet contained some fried and toasted foods.

The present study also has several potential limitations. Most importantly, because of the relatively short intervention duration of 4 weeks, we are unable to draw conclusion on longer-term effects of a diet low or high in AGEs. However, in line with the current data, we recently showed no associations between habitual intake of dietary AGEs, assessed by an FFQ with a reference period of one year, and arterial stiffness and generalized microvascular function in a population-based cohort[38,57]. Another limitation is that the habitual intake of AGEs, as assessed by an FFQ, was higher in our low AGE group than our high AGE group, but this occurred by chance as almost all other outcomes showed no imbalance at baseline. However, this imbalance is expected to enhance the dietary effects, due to the greater differences with the habitual diet. Additionally, participants were abdominally obese but otherwise healthy White West-Europeans, and extrapolating our findings to groups with other metabolic characteristics or individuals of other ethnicities should be done with caution. Specifically, whether short-term modulation of AGE intake influences the current outcomes in those with diabetes of impaired kidney function cannot be deduced from the current study. Also, we cannot exclude the possibility that disparities in micronutrients between diets may have confounded our results. However, regarding vitamins, the difference in daily intake between groups is far less than dosages used in intervention trials in which these vitamins were associated with health improvements[79, 80]. Moreover, both intervention diets were constructed to contain the recommended daily requirements for all micronutrients. Furthermore, as dietary AGEs are derived from whole foods, a low or high AGE diet will always be accompanied by disparities in some nutrients. Also, we did not monitor physical activity during the intervention period. Although all participants were instructed not to alter their physical activity pattern, increased awareness of dietary habits during the intervention could lead to short-term changes in lifestyle. However, if this occurred, we have no reason to suspect why this would affect one intervention group more than the other. Lastly, although the intervention diets were designed to differ in AGEs, dicarbonyls, reactive precursors to AGEs[81], were also lower in the low compared to the high AGE diet, albeit to a much lesser extent. As such, we cannot

exclude that dietary dicarbonyls contributed to the present findings. Due to their common source (the Maillard reaction), future studies reporting health effects of either dietary AGEs or dicarbonyls should consider this as well.

In conclusion, we provide the most extensive overview to date that a 4-week diet low or high in AGEs has no effect on insulin sensitivity, secretion, and clearance, as well as vascular function and overall inflammation in abdominally obese but healthy individuals. These findings require validation in large prospective cohort studies and in different populations with established disease such as diabetes and kidney failure.

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Supplementary Methods

Macrocirculation

A 3-lead electrocardiogram (ECG) was recorded continuously during the measurements to facilitate automatic signal processing. During arterial stiffness measurement, repeated brachial systolic, diastolic, and mean arterial pressures were obtained at 5-minute intervals, using an oscillometric device (Accutorr Plus, Datascope, Inc, Montvale, NJ). The time-averages of systolic, diastolic, and mean arterial pressure were used in the analysis.

Carotid-to-femoral Pulse Wave Velocity

Aortic stiffness was determined by measuring carotid-to-femoral pulse wave velocity (cfPWV) according to recent guidelines[1] with the use of applanation tonometry (SphygmoCor, Atcor Medical, Sydney, Australia). Pressure waveforms were determined at the left common carotid and left common femoral arteries. The difference in the time of pulse arrival from the R-wave of the ECG between the 2 sites (transit time) was determined with the intersecting tangents algorithm. The pulse wave travel distance was calculated as 80% of the direct straight distance (measured with an infantometer) between the 2 arterial sites. cfPWV was defined as travelled distance/transit time. We used the median of 3 consecutive cfPWV recordings in the analyses. When more than 3 measurements were performed, the measurements with the lowest standard deviation were used.

Carotid Distensibility coefficient and Young's elastic modulus

Indices of carotid stiffness were measured at the left common carotid artery (10 mm proximal to the carotid bulb), with the use of an ultrasound scanner equipped with a 7.5-MHz linear probe (MyLab 70, Esaote Europe B.V., Maastricht, the Netherlands). This set-up enables the measurement of diameter, distension, and intima-media thickness (IMT) as described previously[2, 3]. Briefly, during the ultrasound measurements, a B-mode image based on 19 M-lines was displayed on screen. An online echo-tracking algorithm showed real-time anterior and posterior wall displacements. The multiple M-line recordings were composed of 19 simultaneous recordings at a frame rate of 498 Hz. The distance between the M-line recording positions was 0.96 mm; thus, a total segment of 18.24 mm of each artery was covered by the scan plane. For offline processing, the radiofrequency signal was acquired by a dedicated computer-based system (ART.LAB, Esaote Europe B.V. Maastricht, the Netherlands) with a sampling frequency of 50 MHz. Data processing

was performed in MatLab (version 7.5; Mathworks, Natick, Massachusetts, USA). Distension waveforms were obtained from the radiofrequency data by wall tracking, as described in[2]. We defined carotid IMT as the distance of the posterior wall from the leading edge interface between lumen and intima to the leading edge interface between media and adventitia[3]. We used the median diameter, median distension and median IMT of three recordings in the analyses.

Data analysis was done by quantifying the local arterial elastic properties through the calculation of the following indices[4]:

1. Distensibility coefficient (carDC) = (2ΔD × D + ΔD2)/(PP × D2) (10–3/kPa)
2. Young's elastic modulus (carYEM) = D/(IMT × distensibility coefficient) (103 kPa)

where D is the arterial diameter; ΔD is the distension; IMT the intima–media thickness; and PP the pulse pressure. Local carotid PP was estimated according to the calibration method described by Kelly and Fitchett[5], with the use of carotid tonometry waveforms as adapted by van Bortel et al.[6]. This method assumes a constant difference between MAP and diastolic pressure along the arterial tree. PP can then be calculated at a carotid artery (PPcar) from the uncalibrated carotid pressure waveform using the formula: PPcar = PPcaruncalibrated × (Kbrach/Kcaruncalibrated), in which K is defined as (MAP − diastolic pressure). For the carotid artery, diastolic pressure and MAP are calculated as the minimum and the area under the tonometry waveform divided by time, respectively. The carDC reflects the inverse of arterial stiffness at operating pressure. The carYEM reflects the stiffness of the arterial wall material at operating pressure.

Note that increased values of cfPWV or of carYEM, or decreased values of carDC, indicate increased central arterial stiffness.

Radial Pulse Wave Analysis

Radial artery pulse wave analysis was measured in triplicate at the wrist of the right arm using tonometry (SphygmoCor v9; AtCor Medical), as described previously[7]. In short, the central arterial waveform was derived from the peripheral arterial waveform using a validated transfer function. The augmentation index was defined as the difference between the first and second peak of the central arterial waveform, expressed as a percentage of the pulse pressure and corrected for heart rate. We used the median of 3 consecutive measurements. As in some participants more than 3 measurements were performed, we chose the measurements with the highest

quality based on four criteria[8]: (1) average pulse height above 100 units, (2) pulse height variation < 5%, (3) diastolic variation < 5%, and (4) systolic peak between 80 and 150 ms from the start of the wave. Measurements were scored on a scale from 0 to 4 based on the number of criteria met. Measurements with low quality scores (0 or 1) were excluded from the analysis. For one participant, this resulted in 2 measurements instead of 3, and these 2 measurements were averaged instead of using the median.

Flow-mediated dilation

Flow-mediated dilation (FMD) of the brachial artery was assessed by ultrasound echography in dual mode (MyLab70, Esaote) and recording of echo images on DVD, as described previously[7]. These images were analyzed offline using a custom-written Matlab program (MyFMD; AP Hoeks, Department of Biomedical Engineering, Maastricht University Medical Center, Maastricht, the Netherlands). After a 5-minute reference period, a pneumatic cuff placed around the participant's right forearm was inflated to 200 mmHg for 5 minutes to ensure arterial occlusion. After 5 minutes of arterial occlusion, the cuff was deflated and images were obtained for an additional 5 minutes. The FMD response was quantified as the maximal percentage change in post occlusion arterial diameter relative to the baseline diameter.

Microcirculation

Laser Doppler Flowmetry

Skin blood flow was measured both in the basal state, during acute hyperinsulinemia, and during acute local heating, as described previously, by means of a laser-Doppler system (Periflux 5000; Perimed, Järfalla, Sweden) equipped with two thermostatic laser-Doppler probes (PF457; Perimed) at the dorsal side of the wrist of the left hand[9]. The laser-Doppler output was recorded for 35 minutes with a sample rate of 32 Hz, which gives semi quantitative assessment of skin blood flow expressed in arbitrary perfusion units.

Flowmotion

Since skin microvascular flowmotion (SMF) has predominantly been observed in participants with a skin temperature above 29.3°C[10], the laser-Doppler probe was set at 30°C. The skin blood flow signal was transformed into five different SMF components by means of a Fast-Fourier transform algorithm using dedicated custom build

software (FlowPSD; AP Hoeks, Department of Biomedical Engineering, Maastricht University Medical Center, Maastricht, the Netherlands). The frequency spectrum between 0.01 and 1.6 Hz was divided into five components: (1) endothelial, 0.01-0.02 Hz, (2) neurogenic, 0.02-0.06 Hz, (3) myogenic, 0.06-0.15 Hz, (4) respiratory, 0.15-0.40 Hz, and (5) heartbeat, 0.40-1.60 Hz[11]. Additionally, total SMF energy was obtained by the sum of the power density values of the total frequency spectrum.

Heat-induced skin hyperemic response

With the second probe, skin blood flow was first recorded unheated for 2 minutes to serve as a baseline. After the 2 minutes of baseline, the temperature of the probe was rapidly and locally increased to 44°C and was then kept constant until the end of the registration. The heat-induced skin hyperemic response was expressed as the percentage increase in average perfusion units during the 33-minute heating phase over the average baseline perfusion units.

Retinal imaging

Fundus photographs were obtained to assess static retinal microvascular diameters with a non-mydriatic manual-focus fundus camera (Canon). To this end, three optic-disc centered photographs of the right eye were taken. The detailed procedure has been explained elsewhere[12]. In short, retinal arteriolar and venular diameters were measured at an area 0.5-1.0 disc diameter away from the optic disc margin with semi-automatic analyzing software (Vesselmap 3.0, Visualis, Imedos Systems UG). Arteriolar and venular diameters were averaged to central retinal arteriolar (CRAE) and venular (CRVE) equivalents using the Parr-Hubbard formula[13]. Vessel diameters are presented in µm, as one measuring unit of the imaging device relates to 1 µm in the model of Gullstrand's normal eye. The same researcher took all images, and all images were analysed by the same independent researcher, unaware of a participant's treatment allocation. Participants with retinal pathologies that influence microvascular calibers (e.g. macular degeneration, n = 1) were excluded from the analyses.

Habitual food intake and dietary advanced glycation endproducts

We assessed habitual dietary intake by a validated 253-item food frequency questionnaire (FFQ)[14]. This FFQ contains 101 questions on consumption with a reference period of one year. The FFQ collected information on the intake of major food groups. Food intake was determined by the combination of frequency

questions with quantity questions. For the frequency questions, 11 options were available ranging from "not used" to 7 days/week. For the quantity questions, variable options were available based on fourteen standard household servings, ranging from < 1/day to > 12/day. Average daily consumption of food items was then calculated by multiplying the frequency and amount. Energy and nutrient intakes were subsequently determined by transcribing food items into food codes embedded in the Dutch Food Composition Table 2011[15]. Additionally, we determined the Dutch Healthy Diet (DHD) index based on this food intake data. The DHD-index is a measure of diet quality as it assesses adherence to the Dutch dietary guidelines[16]. A higher index has been associated with more nutrient-dense diets and lower risk of mortality[17, 18].

Dietary AGE intake was determined by coupling the consumption of food items within the FFQ to our dietary AGE database[19]. In this database, three major AGEs, CML, CEL, and MG-H1, were quantified in protein fractions of food products using highly specific ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). In total, this database includes over 200 food products commonly consumed in a Western diet. For each participant, AGE intake was estimated as described previously[20]. Some of the food products in the FFQ were not analyzed for AGEs content. AGE content of these specific products were estimated by matching them to other products that were comparable in macronutrient profile and preparation method. For example, for several fresh vegetables boiled in water, such as endive, beets, leek, and spinach, the same AGE content was used. By comparison, jarred peas and carrots were measured separately from fresh peas and carrots, as AGEs in jarred peas and carrots are higher as they contain added sugar and are heated to prolong shelf life[19].

Skin autofluoresence

Skin autofluoresence (SAF) was measured with the AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands). The AGE reader is a desktop device that uses the characteristic fluorescent properties of certain AGEs to estimate the level of AGE accumulation in the skin. Technical details of this noninvasive method have been described more extensively elsewhere[21]. In short, the AGE Reader illuminates a skin surface of 4 cm^2 guarded against surrounding light, with an excitation wavelength range of 300 to 420 nm, with a peak excitation of 370 nm. SAF was calculated as the ratio between the emission light from the skin in the wavelength range of 420 to 600 nm (fluorescence) and excitation light that is reflected by the skin (300–420 nm), multiplied by 100 and expressed in arbitrary units. Participants were asked not to use

any sunscreen or self-browning creams on their lower arms within 2 days before the measurement. SAF was measured at room temperature in a semidark environment, where participants were at rest in a seated position. The inner side of the forearm ≈ 4 cm below the elbow fold of a participant was positioned on top of the device, as described by the manufacturer. The mean of 3 consecutive measurements was used in the analyses.

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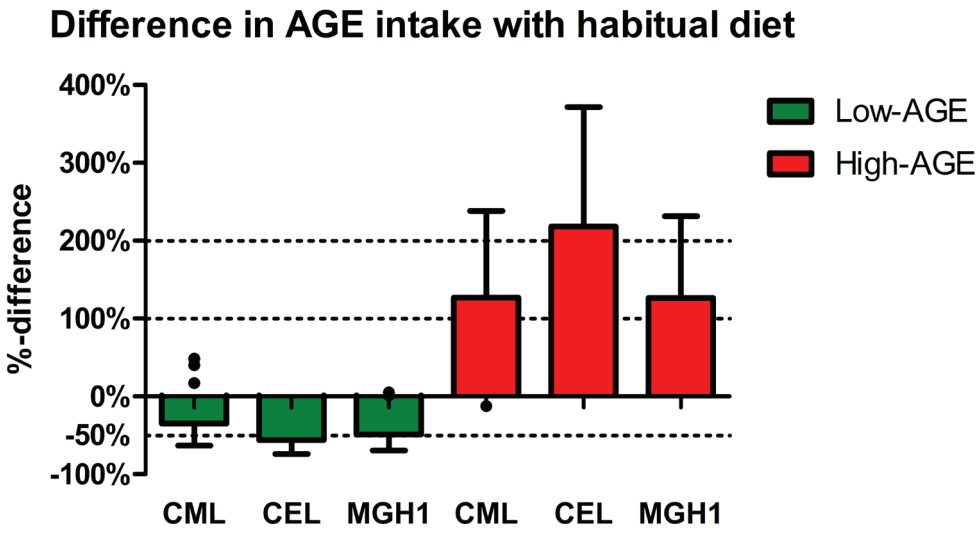
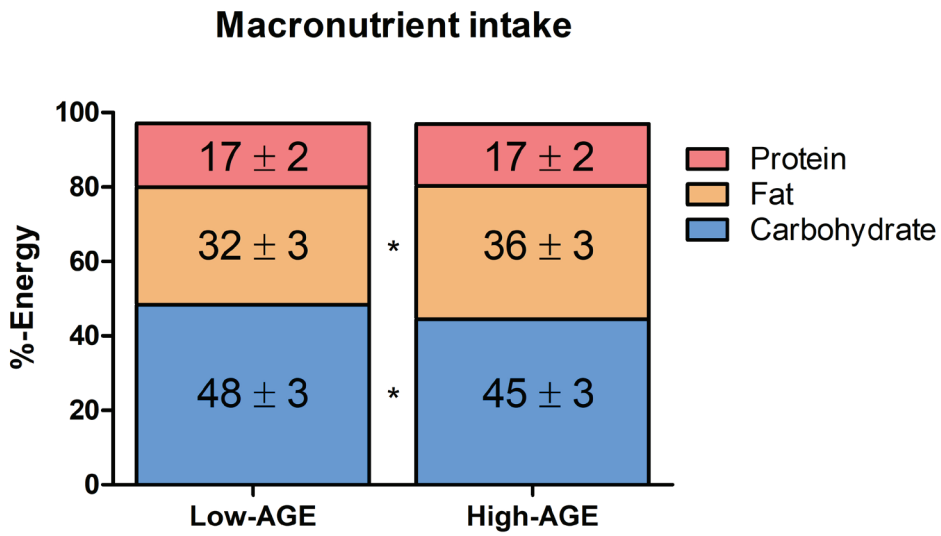
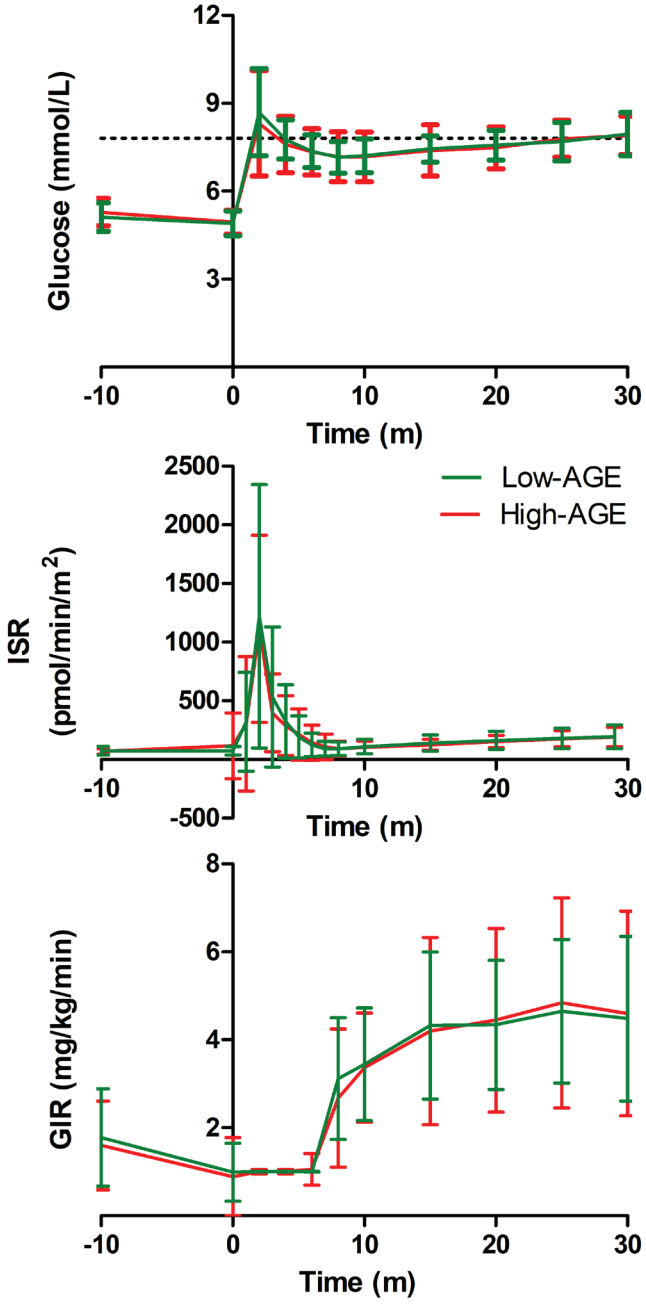
Supplementary Figures



Supplementary Figure 6.1 Percentage difference in intake of AGEs between the habitual diet (assessed with food frequency questionnaires) and during the intervention (assessed as the average daily intake from two five-day dietary logs). Bar plots indicate mean ± SD. Black circles indicate individuals that showed no increase or decrease in respective AGE intake during their intervention. *n* = 34 for the low AGE group, *n* = 38 for the high AGE group.



Supplementary Figure 6.2 Difference in macronutrient intake (as percentage-energy) during the low and high AGE diet. * indicates a difference between intervention diets at the < 0.05 level. Fiber intake represented 2%, while alcohol intake represented 1% of percentage-energy in both groups.



Supplementary Figure 6.3 Hyperglycemic clamp results at follow-up (*n* = 72). Upper: plasma glucose after administration of the glucose bolus and subsequent clamping at 2.8 mmol/L above fasting values (dotted line). Middle: ISR profile. Lower: Glucose infusion rate. Abbrevations: GIR: glucose infusion rate. ISR: insulin secretion rate.

Supplementary Tables

Supplementary Table 6.1 Comparison of baseline characteristics from participants included in the complete case analysis to those excluded resulting from missing the primary outcome.

Characteristic	Primary outcome at follow-up not collected (*n* = 9)	Primary outcome at follow-up collected (*n* = 73)
Demographics[1]		
Age (years)	46 ± 19	52 ± 14
Males/Females	1/8	22/51
Weight (kg)	89.1 ± 3.6	88.3 ± 13.7
Waist circumference (cm)		
Men	108.0 ± 0.0	107.3 ± 5.9
Women	99.5 ± 6.7	100.6 ± 8.2
BMI (kg/m^2)	29.8 ± 2.8	30.6 ± 4.0
24-hour systolic BP[2] (mmHg)	112 ± 12	125 ± 11
24-hour diastolic BP[2] (mmHg)	75 ± 6	78 ± 8
Biological		
Fasting glucose (mmol/L)	5.3 ± 0.36	5.0 ± 0.5
Fasting insulin (pmol/L)	8.3 ± 0.9	10.1 ± 4.2
HbA1c[1] (%)	5.3 ± 0.4	5.3 ± 0.3
HDL cholesterol (mmol/L)	1.4 ± 0.25	1.4 ± 0.4
LDL cholesterol (mmol/L)	3.4 ± 1.1	3.5 ± 0.8
Triglycerides (mmol/L)	1.4 ± 0.8	1.4 ± 0.7
Fatty liver index (unitless)	57 ± 25	61 ± 22
eGFR (ml/min/1.73m^2)	86.4 ± 21.5	89.1 ± 16.0
Habitual dietary intake[3]		
Energy intake (kcal/day)	2116 ± 476	2286 ± 814
Dutch Healthy Diet index	73.7 ± 12.7	82.1 ± 15.6
CML (mg/day)	3.23 ± 0.33	4.07 ± 1.71
CEL (mg/day)	3.00 ± 0.28	3.83 ± 1.77
MG-H1 (mg/day)	21.34 ± 4.56	27.03 ± 10.46
MGO (mg/day)	3.07 ± 0.43	3.73 ± 1.51
GO (mg/day)	2.93 ± 0.53	3.59 ± 1.441
3-DG (mg/day)	9.97 [7.41,-]	15.00 [10.84,25.94]
Primary outcomes		
Insulin sensitivity[1] (mg/kg/min)	6.7 ± 4.4	4.4 ± 1.9
First-phase insulin secretion[3] (pmol/min/m^2)	161 ± 88	263 ± 155
IMMR[4] (%)	-	7 ± 36

Data are presented as means ± SD, medians [interquartile range], or percentages. Abbreviations: 3-DG: 3-Deoxyglucose. BP: blood pressure. CEL: N$^{\varepsilon}$-(1-carboxyethyl)lysine. CML: N$^{\varepsilon}$-(carboxymethyl)lysine. eGFR: estimated glomerular filtration rate. GO: Glyoxal. IMMR: Insulin-mediated microvascular recruitment. MGO: Methylglyoxal. MG-H1: N$^{\delta}$-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. [1] *n* = 7 for those missing, *n* = 73 for those included. [2] *n* = 7 for those missing, *n* = 71 for those included. [3] *n* = 3 for those missing, *n* = 72 for those included. [4] *n* = 0 for those missing, *n* = 72 for those included.

Supplementary Table 6.2 Average daily AGE-, dicarbonyl- and energy- intake during the intervention as determined by two 24-hour recalls

Nutrient	Low AGE (*n* = 37)[1]	High AGE (*n* = 37)	Low vs High *p*
AGEs (mg/day)			
CML	2.67 ± 0.96	6.66 ± 3.40	**<0.001**
CEL	1.65 ± 0.68	8.31 ± 7.20	**<0.001**
MG-H1	13.72 ± 3.78	47.70 ± 20.84	**<0.001**
Dicarbonyls (mg/day)			
MGO	2.85 ± 0.93	3.66 ± 1.12	**0.001**
GO	2.87 ± 0.90	3.27 ± 0.77	**0.027**
3-DG	14.22 ± 7.04	17.42 ± 9.42	0.231
Energy (kcal/day)			
Energy intake[1]	2050 ± 500	2162 ± 621	0.367

Daily intakes (means ± SD, medians [IQR]) were assessed from two 24-hour recalls in week 3 and week 4 of the intervention. Differences between intervention groups were tested by a one-factor ANCOVA with energy intake, sex, and age as covariates. [1]Energy intake was not included as a covariate.

Supplementary Table 6.3 Average daily micronutrient intake during the low and high AGE diets

Micronutrient	Low AGE (*n* = 34)[1]	High AGE (*n* = 38)	Low vs High *p*
Vitamins			
Retinol activity equivalents (µg/day)	1579.4 ± 547.2	534.7 ± 172.4	**<0.001**
Thiamine (mg/day)	1.1 ± 0.2	1.1 ± 0.2	**0.039**
Nicotinic acid (mg/day)	17.8 ± 3.8	18.2 ± 4.7	0.179
Pyridoxin (mg/day)	1.5 ± 0.3	1.3 ± 0.3	**<0.001**
Folate equivalents (µg/day)	225.5 ± 34.1	224.1 ± 51.1	**0.026**
Cobalamin (µg/day)	4.8 ± 0.9	4.4 ± 1.0	**<0.001**
Ascorbic acid (µg/day)	67.9 ± 16.3	73.9 ± 16.4	0.298
Cholecalciferol (µg/day)	4.1 ± 1.3	5.4 ± 2.1	**0.005**
Calcidiol (µg/day)	0.3 ± 0.2	0.3 ± 0.1	0.758
Tocopherols and tocotrienols (mg/day)	9.1 ± 1.8	12.3 ± 1.8	**<0.001**
Minerals			
Calcium (mg/day)	1031.9 ± 172.8	1002.3 ± 230.9	**0.014**
Phosphor (mg/day)	1496.5 ± 257.1	1616.2 ± 380.2	0.097
Total iron (mg/day)	11.3 ± 3.1	11.5 ± 3.0	0.208
Sodium (mg/day)	2286.2 ± 464.3	2647.4 ± 741.2	**<0.001**
Potassium (mg/day)	3252.8 ± 652.1	3444.7 ± 721.6	0.781
Magnesium (mg/day)	331.6 ± 70.0	385.1 ± 96.1	**<0.001**
Zink (mg/day)	10.7 ± 2.0	10.4 ± 2.5	**<0.001**
Selenium (µg/day)	47.7 ± 8.4	53.5 ± 15.3	**0.041**
Copper (mg/day)	1.2 ± 0.3	1.5 ± 0.4	**<0.001**
Iodine (µg/day)	211.7 ± 43.2	200.5 ± 53.1	**<0.001**

Daily intakes (means ± SD, medians [IQR]) of micronutrients were assessed from two five-day dietary logs at week 1 and week 4 of the intervention. Differences between intervention groups were tested by a one-factor ANCOVA with energy intake, sex, and age as covariates. [1]Dietary logs were not returned by one participant in the low AGE group.

Supplementary Table 6.4 Effects of a 4-week low and high AGE diet on AGEs and oxoaldehydes in urine and plasma of abdominally obese individuals

Variable	Low AGE (*n* = 36)				High AGE (*n* = 38)				Low compared to High	
	Baseline	4 Week	Delta	*p*	Baseline	4 Week	Delta	*p*	Overall difference*	*p*
Urine (nmol/mmol/creatinin)										
CML[1]	936.4 ± 221.6	949.1 ± 292.6	12.7 ± 255.2	0.77	980.3 ± 292.6	1101.7 ± 412.0	121.5 ± 399.6	0.07	-124.9 [-276.0,26.2]	0.10
CEL[1]	493.6 ± 138.5	476.9 ± 140.9	-16.8 ± 130.8	0.45	470.5 ± 121.5	762.7 ± 236.0	**292.2 ± 260.2**	**<0.01**	**-286.2 [-376.5,-195.9]**	**<0.01**
MG-H1[1]	2343 ± 1196	1902 ± 1061	-441.1 ± 1454	0.08	2399 ± 1218	4452 ± 1958	**2053 ± 2286**	**<0.01**	**-2460 [-3188,-1731]**	**<0.01**
Pyrraline[1]	1045 ± 369.6	1267 ± 554.6	**222.2 ± 424.2**	**<0.01**	991.9 ± 376.7	2142 ± 1028	**1151 ± 1105**	**<0.01**	**-914.4 [-1304,-525.4]**	**<0.01**
MGO	128.0 ± 103.1	112.0 ± 40.2	-15.9 ± 79.4	0.24	110.2 ± 46.9	121.6 ± 64.6	11.5 ± 53.4	0.19	-15.2 [-36.8,6.3]	0.16
8-oxo-dG	1.4 ± 0.9	1.4 ± 0.9	0.0 ± 0.6	0.77	1.6 ± 1.4	1.4 ± 1.1	-0.2 ± 1.4	0.48	0.00 [-0.4,0.4]	0.98
CeDG (log)	-1.9 ± 0.8	-2.2 ± 0.6	**-0.3 ± 0.7**	**0.03**	-1.8 ± 1.9	-1.2 ± 0.	**0.7 ± 1.2**	**<0.01**	**-1.0 [-1.3,-0.6]**	**<0.01**
Plasma (nmol/L)										
Free CML[1]	87.4 ± 27.7	82.1 ± 27.4	-5.3 ± 31.6	0.32	88.1 ± 35.7	97.0 ± 26.5	**8.8 ± 27.4**	**0.05**	**-12.5 [-22.8,-2.2]**	**0.02**
Free CEL[1]	43.8 ± 11.3	40.2 ± 9.9	-3.6 ± 11.3	0.07	44.3 ± 16.7	81.9 ± 33.7	**37.6 ± 31.3**	**<0.01**	**-39.5 [-50.1,-28.9]**	**<0.01**
Free MG-H1[1]	95.7 ± 63.1	77.0 ± 32.0	-18.7 ± 61.9	0.08	105.9 ± 63.1	221.6 ± 122.6	**115.7 ± 110.6**	**<0.01**	**-133.2 [-170.0,-96.4]**	**<0.01**
MGO	309.1 ± 42.2	309.6 ± 44.3	-1.8 ± 52.8	0.84	309.1 ± 42.2	307.2 ± 41.9	-7.3 ± 42.5	0.30	0.9 [-17.9,19.7]	0.92
GO	443.0 ± 115.4	400.0 ± 89.6	-43.3 ± 131.8	0.06	445.1 ± 100.9	422.5 ± 100.5	-22.7 ± 101.1	0.18	-18.5 [-60.2,23.2]	0.38
3-DG	1059 ± 96.2	1066 ± 100.4	6.6 ± 65.2	0.55	1114 ± 117.9	1105 ± 79.8	-9.2 ± 65.4	0.39	-2.7 [-27.8,22.5]	0.83
Skin (arbitrary units)										
Autofluoresence (SAF)	1.99 ± 0.39	1.96 ± 0.41	-0.03 ± 0.22	0.34	2.03 ± 0.32	2.03 ± 0.38	0.00 ± 0.24	0.98	-0.04 [-0.14,0.07]	0.51

Values are presented as means ± SD. Within-group changes were evaluated with a paired-samples t test. Overall differences after the low compared to high AGE diet were evaluated with a one-way ANCOVA with adjustment for age, sex, and the baseline variable of interest. Abbreviations: 3-DG: 3-Deoxyglucose. 8-oxo-dG: 8-Oxo-2'-deoxyguanosine. CEDG: N^2-(1-carboxyethyl)-2'-deoxyguanosine. CEL: N^{ε}-(1-carboxyethyl)lysine. CML: N^{ε}-(carboxymethyl)lysine. GO: Glyoxal. MGO: Methylglyoxal. MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine. Pb: Protein-bound. [1]*n* = 35 for low AGE group due to exclusion of a non-compliant participant.

Supplementary Table 6.5 Effects of a 4-week low and high AGE diet on outcomes of glucose metabolism of abdominally obese individuals

Variable	Low AGE (*n* = 36)				High AGE (*n* = 38)				Low compared to High	
	Baseline	4 Week	Delta	*p*	Baseline	4 Week	Delta	*p*	Overall difference	*p*
Fasting indices										
Fasting glucose (mmol/L)	4.87 ± 0.44	4.95 ± 0.44	0.08 ± 0.27	0.08	5.09 ± 0.49	5.04 ± 0.39	-0.04 ± 0.28	0.36	0.06 [-0.05,0.18]	0.29
Fasting insulin[1] (µIU/ml)	9.50 ± 2.83	9.88 ± 3.50	0.38 ± 2.35	0.34	10.65 ± 5.10	10.34 ± 3.75	-0.31 ± 4.10	0.64	0.17 [-1.19,1.53]	0.80
Fasting c-peptide[1] (pg/L)	1554.4 ± 509.6	1586.3 ± 548.0	31.9 ± 290.3	0.52	1607.9 ± 548.0	1598.1 ± 491.8	-9.8 ± 279.1	0.83	34.7 [-96.5,165.9]	0.60
Insulin sensitivity										
Insulin sensitivity[1] (mg/kg/min)	4.53 ± 1.84	4.58 ± 1.91	0.05 ± 1.74	0.86	4.53 ± 1.84	4.66 ± 2.10	0.38 ± 1.46	0.12	-0.25 [-0.96,0.47]	0.49
M/I[1]	4.97 ± 2.57	4.90 ± 2.61	-0.07 ± 2.08	0.85	4.67 ± 2.88	4.91 ± 2.66	0.23 ± 1.59	0.37	-0.20 [-1.01,0.61]	0.63
HOMA (µU·L/mmol·L)	2.09 ± 0.75	2.21 ± 0.84	0.12 ± 0.57	0.23	2.41 ± 1.18	2.33 ± 0.95	-0.08 ± 0.90	0.60	0.07 [-0.24,0.39]	0.65
Insulin clearance										
Fasting insulin clearance[1] (L/min/m^2)	1.14 ± 0.19	1.13 ± 0.18	-0.01 ± 0.15	0.69	1.13 ± 0.31	1.10 ± 0.24	-0.03 ± 0.18	0.37	0.02 [-0.04,0.09]	0.49
Steady state insulin clearance[2] (L/min/m^2)	0.49 ± 0.10	0.48 ± 0.09	-0.01 ± 0.06	0.21	0.49 ± 0.10	0.49 ± 0.08	-0.01 ± 0.08	0.54	-0.01 [-0.04,0.02]	0.62
Insulin secretion										
Fasting insulin secretion[1] (pmol/min/m^2)	66 ± 20	67 ± 21	1 ± 12	0.58	66 ± 20	66 ± 19	-0 ± 12	0.86	1 [-4,7]	0.66
C-peptide suppression[2] (%)	-13.4 ± 37.7	-6.2 ± 32.1	7.2 ± 36.3	0.26	-9.3 ± 36.8	-1.1 ± 40.1	8.2 ± 28.3	0.08	-3.4 [-17.2,10.4]	0.62
Glucose increment $t_{0\text{-}8}$ minutes[3] (mmol/L)	2.79 ± 0.68	2.85 ± 0.62	0.06 ± 0.59	0.56	2.94 ± 0.83	2.66 ± 0.86	-0.29 ± 0.89	0.06	0.26 [-0.06,0.58]	0.12
1st phase ISR[3] (pmol/min/m^2)	264 ± 161	285 ± 181	21 ± 68	0.08	265 ± 154	267 ± 150	1 ± 77	0.94	17 (-18,51)	0.33
2nd phase ISR[3] (pmol/min/m^2)	150 ± 60	157 ± 73	8 ± 47	0.33	150 ± 50	148 ± 53	-2 ± 37	0.76	11 (-9,30)	0.28
2nd phase ISR steady state[3] (pmol/min/m^2)	179 ± 66	188 ± 92	9 ± 64	0.44	176 ± 61	184 ± 74	8 ± 53	0.35	1 (-26,28)	0.95
β-GS[3] (pmol/min/m^2/mmol·L)	41 ± 19	40 ± 25	-1 ± 19	0.82	39 ± 18	43 ± 24	4 ± 22	0.25	-5 (-15,5)	0.32

Values are presented as means ± SD. Within-group changes were evaluated with a paired-samples t test. Overall differences after the low compared to high AGE diet were evaluated with a one-way ANCOVA with adjustment for age, sex, and the baseline variable of interest. Abbrevations: β-GS: beta-cell glucose sensitivity. ISR: Insulin secretion rates. M/I: insulin sensitivity adjusted for plasma insulin. [1] *n* = 35 for low AGE, *n* = 38 for high AGE. [2] *n* = 34 for low AGE, *n* = 38 for high AGE. [3] *n* = 34 for low AGE, *n* = 36 for high AGE.

Supplementary Table 6.6 Effects of a 4-week low and high AGE diet on micro and macrovascular function of abdominally obese individuals

Variable	Low AGE (*n* = 36)				High AGE (*n* = 38)				Low compared to High	
	Baseline	4 Week	Delta	*p*	Baseline	4 Week	Delta	*p*	Overall difference	*p*
Microvascular function										
IMMR[1] (%)	8.8 ± 41.5	7.6 ± 39.3	-1.2 ± 55.4	0.90	5.0 ± 31.6	10.6 ± 28.4	5.5 ± 43.1	0.43	-3.1 [-19.5,13.4]	0.71
Skin heating response (%)	1309.5 ± 645.7	1268.6 ± 733.2	-40.9 ± 821.4	0.77	1444.8 ± 820.6	1251.4 ± 772.7	-193.5 ± 784.5	0.14	33.5 [-292.2,359.1]	0.84
CRAE[2] (µm)	174.4 ± 15.7	173.9 ± 16.1	-0.5 ± 7.9	0.74	174.1 ± 18.7	171.7 ± 17.7	**-2.5 ± 6.5**	**0.03**	1.9 [-1.7,5.5]	0.30
CRVE[2] (µm)	213.7 ± 13.3	212.3 ± 15.2	-1.4 ± 5.7	0.21	217.0 ± 19.0	212.7 ± 16.7	**-4.3 ± 7.3**	**<0.01**	1.6 [-2.1,5.2]	0.39
Plasma biomarker of endothelial dysfunction Z-score[3] (SD)	-0.09 ± 1.00	-0.03 ± 1.11	0.06 ± 0.57	0.53	0.09 ± 1.00	0.03 ± 0.89	-0.06 ± 0.52	0.51	0.11 [-0.13,0.35]	0.38
sICAM-1[3] (ng/ml)	350.18 ± 78.66	335.08 ± 77.88	**-15.09 ± 44.03**	**0.05**	375.09 ± 111.92	361.26 ± 82.96	-13.83 ± 54.69	0.13	-7.50 [-26.65,11.65]	0.44
sVCAM-1[3] (ng/ml)	400.40 ± 94.21	406.49 ± 95.59	6.08 ± 39.37	0.36	391.39 ± 67.99	393.15 ± 61.99	1.76 ± 33.94	0.75	5.77 [-10.62,22.16]	0.49
eSelectin[3] (ng/ml)	83.41 ± 41.54	76.95 ± 38.34	**-6.46 ± 13.50**	**<0.01**	93.61 ± 48.22	83.09 ± 38.82	**-10.52 ± 19.11**	**<0.01**	2.52 [-3.24,8.28]	0.39
vWF[3] (%)	103.95 ± 35.56	106.37 ± 32.95	2.42 ± 33.36	0.67	107.21 ± 44.44	100.04 ± 41.25	-7.17 ± 27.67	0.12	9.08 [-3.66,21.82]	0.16
Microvascular flowmotion										
Total power signal (arbitrary units)	88613 ± 95195	127765 ± 142863	39151 ± 133479	0.09	107897 ± 105147	170831 ± 368598	62934 ± 396971	0.34	-39966 [-174475,94542]	0.56
Endothelial contribution (%)	54.7 ± 12.0	53.5 ± 11.0	-1.2 ± 12.7	0.58	52.3 ± 11.3	54.9 ± 9.3	2.6 ± 13.6	0.25	-2.3 [-6.9,2.3]	0.33
Myogenic contribution (%)	9.8 ± 5.6	9.7 ±7.3	-0.1 ± 8.3	0.95	11.3 ± 7.9	10.5 ± 6.5	-0.8 ± 10.3	0.62	-0.6 [-3.9,2.7]	0.71
Neurogenic contribution (%)	31.1 ± 7.9	32.9 ± 7.1	1.8 ± 7.1	0.14	31.9 ± 8.2	30.8 ± 6.5	-1.1 ± 7.7	0.37	2.7 [-0.0;5.4]	0.05
Cardiogenic contribution (%)	2.2 ± 2.8	1.8 ± 2.3	-0.4 ± 3.2	0.42	1.8 ± 1.9	1.9 ± 2.1	0.1 ± 1.6	0.79	-0.2 [-1.2,0.7]	0.64
Respiratory contribution (%)	2.1 ± 2.0	2.1 ± 1.7	-0.1 ± 2.6	0.88	2.6 ± 2.9	1.9 ± 2.0	-0.7 ± 1.6	0.07	0.4 [-0.4,1.2]	0.31
Macrovascular function										
FMD[3] (%)	3.5 ± 2.9	3.5 ± 3.0	-0.0 ± 2.2	0.99	3.4 ± 2.8	3.6 ± 3.2	0.16 ± 4.5	0.82	-0.1 [-1.5,1.2]	0.85
cfPWV[3] (m/s)	10.2 ± 2.5	9.7 ± 1.7	-0.6 ± 1.9	0.08	9.9 ± 2.8	10.0 ± 2.7	0.1 ± 1.1	0.76	-0.4 [-1.0,0.2]	0.24
Carotid DC[3] (10^3/kPa)	18.1 ± 9.0	17.8 ± 7.9	-0.3 ± 5.8	0.77	17.4 ± 9.5	16.8 ± 8.4	-0.5 ± 4.5	0.48	0.0 [-2.0,2.0]	0.98
Carotid YEM[3] (10^3kPa)	0.65 ± 0.29	0.63 ± 0.27	-0.02 ± 0.21	0.62	0.64 ± 0.33	0.64 ± 0.27	-0.00 ± 0.20	0.95	0.01 [-0.07,0.09]	0.87
Carotid IMT[3] (um)	787 ± 117	806 ± 145	18 ± 78	0.17	839 ± 137	825 ± 148	14 ± 119	0.46	21 [-24,67]	0.36
Aix[1] (%)	20.8 ± 13.6	21.7 ± 12.6	0.9 ± 0.8	0.26	21.6 ± 10.2	22.0 ± 11.3	0.4 ± 6.6	0.74	0.8 [-1.6,3.2]	0.50
24-h systolic BP[4] (mmHg)	126.5 ± 11.8	125.8 ± 12.0	-0.7 ± 7.4	0.59	123.7 ± 8.8	125.2 ± 10.2	1.6 ± 6.6	0.17	-1.9 [-5.3,1.6]	0.28
24-h diastolic BP[4] (mmHg)	80.4 ± 8.1	79.7 ± 8.8	-0.7 ± 5.0	0.41	80.4 ±8.1	79.7 ± 8.8	0.2 ± 4.7	0.83	-0.2 [-2.5,2.2]	0.87

Values are presented as means ± SD. Within-group changes were evaluated with a paired-samples t test. Overall differences after the low compared to high AGE diet were evaluated with a one-way ANCOVA with adjustment for age, sex, and the baseline variable of interest. Abbreviations: Aix: Augmentation index. bp: blood pressure. Carotid DC: Carotid Distensibility Coefficient. Carotid YEM: Carotid Young's Elastic Modulus. cfPWV: carotid-femoral Pulse Wave Velocity. CRAE and CRVE: Central retinal arteriolar and venular equivalent. FMD: flow mediated dilation. sICAM-1: soluble intracellular adhesion molecule-1. sVCAM-1: soluble vascular adhesion molecule-1. vWF: von Willebrand factor. [1] *n* = 34 for low AGE, *n* = 38 for high AGE. [2] *n* = 30 for low AGE, *n* = 37 for high AGE. [3] *n* = 35 for low AGE, *n* = 38 for high AGE. [4] *n* = 32 for low AGE, *n* = 36 for high AGE.

Supplementary Table 6.7 Effects of a 4-week low and high AGE diet on inflammatory markers and leukocyte differentiation of abdominally obese individuals

Variable	Low AGE (*n* = 35)				High AGE (*n* = 38)				Low compared to High	
	Baseline	4 Week	Delta	*p*	Baseline	4 Week	Delta	*p*	Overall difference	*p*
Inflammatory markers										
Plasma inflammatory markers Z-score (SD)	-0.02 ± 0.95	0.07 ± 1.07	0.09 ± 0.55	0.31	0.02 ± 1.06	-0.07 ± 0.93	0.09 ± 0.58	0.35	0.18 [-0.08,0.44]	0.17
Adiponectin (ug/ml)	14.86 ± 5.44	13.52 ± 4.65	**-1.34 ± 1.61**	**<0.01**	15.00 ± 5.50	15.17 ± 6.04	0.19 ± 1.91	0.55	-1.54 [-2.37,-0.71]	<0.01
MCP-1 (pg/ml)	103.49 ± 27.90	98.53 ± 23.41	-4.95 ± 17.3	0.10	101.45 ± 18.27	99.32 ± 18.12	-2.13 ± 11.3	0.25	-1.01 [-6.89,4.87]	0.73
IL-6 (log)	-0.31 ± 0.65	-0.35 ± 0.56	-0.04 ± 0.43	0.55	-0.31 ± 0.54	-0.43 ± 0.53	-0.11 ± 0.40	0.09	0.07 [-0.11,0.24]	0.46
IL-8 (pg/ml)	3.60 ± 2.92	3.92 ± 3.89	0.32 ± 1.28	0.14	3.35 ± 1.15	3.21 ± 0.84	-0.15 ± 0.87	0.31	0.36 [-0.11,0.82]	0.13
TNFα (pg/ml)	1.16 ± 0.30	1.17 ± 0.28	0.01 ± 0.14	0.64	1.19 ± 0.26	1.16 ± 0.18	-0.04 ± 0.13	0.10	0.04 [-0.02,0.09]	0.16
CRP (log)	0.91 ± 0.92	0.56 ± 0.90	**-0.35 ± 0.70**	**<0.01**	0.72 ± 1.27	0.39 ± 1.02	**-0.33 ± 0.70**	**<0.01**	0.04 [-0.25,0.33]	0.77
SAA (µg/ml)	8.67 ± 10.84	5.92 ± 4.54	-2.75 ± 11.21	0.15	13.71 ± 42.63	5.51 ± 4.37	-8.20 ± 39.30	0.21	0.78 [-0.84,2.40]	0.34
sICAM-1 (ng/ml)	350.18 ± 78.66	335.08 ± 77.88	**-15.09 ± 44.03**	**0.05**	375.09 ± 111.92	361.26 ± 82.96	-13.83 ± 54.69	0.13	-7.50 [-26.65,11.65]	0.44
Leukocyte differentiation										
Leucocytes (10^9/L)	5.92 ± 1.25	5.70 ± 1.16	-0.21 ± 0.66	0.06	6.07 ± 1.47	5.73 ± 1.38	**-0.34 ± 0.65**	**<0.01**	0.10 [-0.19,0.39]	0.49
Segm. Granulocytes (10^9/L)	3.49 ± 1.00	3.28 ± 0.95	**-0.21 ± 0.63**	**0.05**	3.63 ± 1.08	3.38 ± 1.02	**-0.21 ± 0.63**	**0.01**	0.02 [-0.25,0.28]	0.89
Lymphocytes (10^9/L)	1.80 ± 0.49	1.80 ± 0.47	-0.00 ± 0.24	0.99	1.79 ± 0.49	1.74 ± 0.45	-0.05 ± 0.21	0.15	0.04 [-0.05,0.14]	0.38
Monocytes (10^9/L)	0.48 ± 0.12	0.47 ± 0.10	-0.00 ± 0.08	0.82	0.48 ± 0.17	0.43 ± 0.13	**-0.05 ± 0.10**	**<0.01**	**0.05 [0.01,0.08]**	**0.01**
Eosinophils (10^9/L)	0.12 ± 0.05	0.13 ± 0.07	0.01 ± 0.05	0.46	0.15 ± 0.11	0.15 ± 0.09	0.00 ± 0.08	0.96	-0.00 [-0.03,0.03]	0.83
Basophils (10^9/L)	0.05 ± 0.03	0.05 ± 0.02	0.01 ± 0.02	0.09	0.04 ± 0.03	0.05 ± 0.02	0.01	0.12	0.00 [-0.01,0.01]	0.94

Values are presented as means ± SD. Within-group changes were evaluated with a paired-samples t test. Overall differences after the low compared to high AGE diet were evaluated with a one-way ANCOVA with adjustment for age, sex, and the baseline variable of interest. Abbreviations: CRP: C-reactive Protein. MCP-1: IL-6: Interleukin-6, IL-8: Interleukin-8. Monocyte Chemoattractant Protein-1. SAA: Serum Amyloid A. Segm: Segmented. sICAM-1: soluble intracellular adhesion molecule-1. TNFα: Tumor Necrosis Factor alpha.

Supplementary Table 6.8 Effects of a 4-week low and high AGE diet on liver-associated outcomes of abdominally obese individuals

Variable	Low AGE (*n* = 35)				High AGE (*n* = 38)				Low compared to High	
	Baseline	4 Week	Delta	*p*	Baseline	4 Week	Delta	*p*	Overall difference	*p*
Gamma-GT (log)	3.00 ± 0.51	2.99 ± 0.49	-0.02 ± 0.19	0.62	3.13 ± 0.61	3.03 ± 0.58	**-0.10 ± 0.26**	**0.03**	0.07 [-0.04,0.17]	0.19
Triglycerides (mmol/L)	1.14 ± 0.43	1.17 ± 0.40	0.03 ± 0.29	0.51	1.59 ± 0.79	1.49 ± 0.80	**-0.11 ± 0.31**	**0.05**	0.09 [-0.05,0.24]	0.21
LDL-cholesterol (mmol/L)	3.30 ± 0.91	3.22 ± 0.94	-0.08 ± 0.44	0.27	3.72 ± 0.75	3.59 ± 0.91	-0.13 ± 0.49	0.11	0.03 [-0.19,0.25]	0.76
HDL-cholesterol (mmol/L)	1.47 ± 0.41	1.41 ± 0.36	**-0.06 ± 0.15**	**0.02**	1.30 ± 0.29	1.32 ± 0.32	0.02 ± 0.15	0.38	-0.06 [-0.13,0.01]	0.09
Fatty liver index (unitless)	57.35 ± 22.66	55.61 ± 23.69	-1.75 ± 7.17	0.15	63.63 ± 20.58	60.62 ± 21.83	**-3.01 ± 8.14**	**0.03**	1.37 [-2.30,5.03]	0.46

Values are presented as means ± SD. Within-group changes were evaluated with a paired-samples t test. Overall differences after the low compared to high AGE diet were evaluated with a one-way ANCOVA with adjustment for age, sex, and the baseline variable of interest. Abbreviations: Gamma-GT: Gamma-glutamyltransferase.

Supplementary Table 6.9 Multivariate-adjusted associations between indices of AGE intake and outcomes in 72 abdominally obese individuals after a low and high AGE diet

AGEs SD	Plasma Adiponectin mmol/L	Plasma Monocyte Count 10^9/L	Urinary CeDG nmol/mmol kreat	cfPWV m/s	Serum HDL mmol/L	Serum Triglycerides mmol/L
Diet						
CML	**0.85 [0.26,1.44]**	-0.02 [-0.04,0.00]	**0.11 [0.03,0.19]**	0.23 [-0.18,0.64]	**0.05 [0.00,0.10]**	**-0.12 [-0.21,-0.02]**
CEL	**0.82 [0.24,1.39]**	-0.02 [-0.04,0.00]	**0.12 [0.04,0.19]**	0.29 [-0.11,0.69]	**0.05 [0.00,0.09]**	**-0.12 [-0.21,-0.02]**
MG-H1	**0.94 [0.32,1.55]**	-0.02 [-0.05,0.00]	**0.11 [0.02,0.19]**	0.24 [-0.20,0.67]	**0.06 [0.01,0.11]**	**-0.12 [-0.22,-0.01]**
Urine						
CML	**-0.48 [-0.95,-0.02]**	0.00 [-0.01,0.02]	**0.09 [0.03,0.15]**	-0.10 [-0.42,0.22]	-0.01 [-0.05,0.02]	-0.05 [-0.12,0.03]
CEL	0.27 [-0.24,0.78]	-0.01 [-0.03,0.01]	**0.13 [0.07,0.19]**	0.04 [-0.30,0.38]	-0.01 [-0.05,0.03]	**-0.09 [-0.17,-0.02]**
MG-H1	0.08 [-0.44,0.59]	-0.01 [-0.03,0.01]	**0.18 [0.13,0.23]**	-0.11 [-0.45,0.23]	-0.02 [-0.06,0.03]	**-0.08 [-0.16,-0.00]**
Pyrraline	0.16 [-0.34,0.66]	-0.01 [-0.03,0.01]	**0.12 [0.06,0.18]**	0.10 [-0.24,0.44]	-0.02 [-0.06,0.02]	-0.07 [-0.15,0.01]
Plasma						
CML	-0.37 [-0.91,0.16]	-0.02 [-0.04,0.00]	**0.13 [0.07,0.20]**	-0.06 [-0.42,0.31]	0.01 [-0.04,0.05]	0.01 [-0.08,0.09]
CEL	0.11 [-0.42,0.63]	-0.01 [-0.03,0.01]	**0.11 [0.04,0.17]**	-0.13 [-0.48,0.22]	-0.00 [-0.04,0.04]	-0.12 [-0.20,-0.05]
MG-H1	0.04 [-0.50,0.57]	-0.01 [-0.03,0.01]	**0.17 [0.11,0.23]**	-0.22 [-0.59,0.15]	-0.01 [-0.05,0.03]	**-0.12 [-0.20,-0.04]**

Beta's (B) and 95% CIs indicate the difference in outcome per unit change in determinant. Please note that AGEs in diet, urine, and plasma were standardized. Associations were adjusted for age, sex, and intake of carbohydrates, fat, and protein as energy-percentages.

Addendum to Chapter 6

A biomarker for dietary AGE intake

Intake of dietary AGEs is assessed by combining information on food intake, most commonly estimated by food frequency questionnaires (FFQs) or dietary logs, with a database containing AGE content of foods[1-3]. However, methodological limitations of FFQs and dietary logs potentially limit their ability to assess dietary AGE intake accurately. Self-administered semi-quantative FFQs usually estimate food intake over the last month to a year[4-6] and are therefore prone to recall bias when participants incorrectly remember their food intake and portion sizes[7]. Conversely, by estimating food intake with a prospective dietary log based on days to weeks[8, 9] recall bias is prevented but underreporting may occur[10]. A further difficulty is that dietary AGE databases do not contain detailed information on food preparation techniques and duration, which largely determine AGE levels. Although all products in these databases were prepared according to the manufacturer's instructions, an individual may deviate from this. To determine AGE intake reliably in clinical trials, a biomarker is needed. Free AGEs in urine could potentially be used as such, as they are independently associated with dietary AGEs[11]. However, AGEs in urine not only depend on their intake, but also on digestion, uptake, metabolism, excretion, and importantly, endogenous production. Interestingly, the AGE pyrraline, formed from the reaction between 3-deoxy-glucosone and lysine, is suggested by several authors to derive only from dietary sources[12-14]. This is based on the observation that a short-term diet free of cooked, fried, and baked products reduced free urinary pyrraline by 90% in healthy young participants[15]. As such, we explored the potential of using urinary pyrraline levels as a measurement of compliance in our clinical trial where we provide participants with a low and high AGE diet.

To this end, we determined free levels of pyrraline in both 24-hour urine and spot urine samples using UPLC-MS/MS during a sequential 1-day reference period, a 3-day low AGE diet, a 3-day high AGE diet, and a 1-day washout period in a healthy young male.

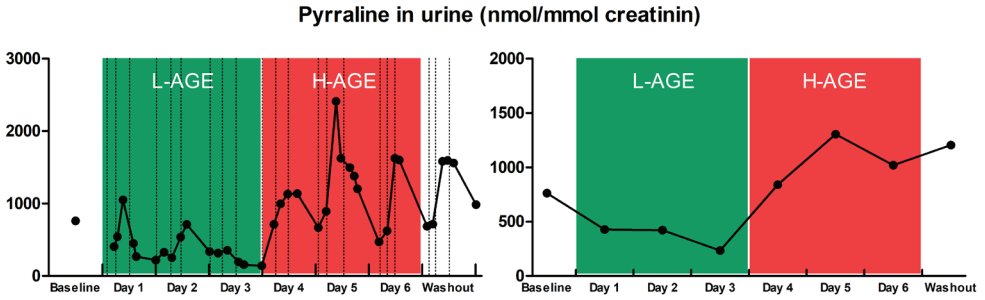


Figure 6.3 Free pyrraline in urine of a healthy young male during several days of a diet low (L-AGE) and high (H-AGE) in AGEs. Left: spot urine samples. Dashed vertical lines indicate breakfast, lunch, and dinner. Right: 24-hour urine samples.

As becomes clear from the spot urine samples (Figure 6.3, left), absorption and subsequent secretion of pyrraline in food occurred rapidly after the meals. In line with this, urinary pyrraline was lowest in fasting first-void samples. However, an overnight fast of approximately 12 hours is not enough to completely normalize pyrraline in urine, as the increase of pyrraline in fasting void urine samples was greater after intake of high AGE meals compared to low AGE meals. Overall, levels of free pyrraline in urine are directly influenced by AGE intake. In the 24-hour urine samples (Figure 6.3, right), we observed a decrease in urinary pyrraline by 69% after three days of the low AGE diet as compared to baseline. Subsequently, after three days of the high AGE diet pyrraline was increased by 336%. These results indicate that free pyrraline in urine is a reflection of dietary AGE intake on the short term, and free pyrraline in 24-hour urine samples may be used as a measurement of compliance to the dietary interventions used in the deAGEing trial. Because of its rapid kinetics, this should still be combined with dietary logs that have a longer reference period. Furthermore, we conclude that dietary AGEs still influence urinary pyrraline levels after an overnight fast of approximately 12 hours. As such, studies investigating AGE levels in urine should standardize dietary intake for at least 24 hours prior to the measurement.

Of note, although it is suggested that pyrraline in urine is derived only from food, there are two studies showing increased levels in individuals with type 1 diabetes comparted to healthy controls[16, 17]. Although these levels were not adjusted for food intake, endogenous production of pyrraline cannot be ruled out. Additionally, its urinary excretion may differ in those with kidney disease[18]. As such, comparing levels of urinary pyrraline should be confined to individuals with similar glucose metabolism status and kidney function.

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dietary AGEs in PEANUT BUTTER and CHOCOLATE SPRINKLES SANDWICH
CML: 8.1; CEL 9.2; MG-H1: 57.3 mg/100g

Chapter 7

A 4-Week Diet Low or High in Advanced Glycation Endproducts Has Limited Impact on Gut Microbial Composition in Abdominally Obese Individuals: The deAGEing Trial

Armand M.A. Linkens, Niels van Best, Petra M Niessen, Nicole E.G. Wijckmans, Erica E.C. de Goei, Jean L.J.M. Scheijen, Martien C.J.M. van Dongen, Christel C.J.A.W. van Gool, Willem M. de Vos, Alfons J.H.M. Houben, Coen D.A. Stehouwer, Simone J.M.P. Eussen, John Penders, and Casper G. Schalkwijk

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Abstract

Background: Dietary advanced glycation endproducts (AGEs), abundantly present in Westernized diets, are linked to negative health outcomes, but their impact on the gut microbiota has not yet been well investigated in humans.

Objective: To determine the effect of a diet low and high in AGEs on gut microbial composition.

Methods: We investigated the effects of a 4-week isocaloric and macronutrient-matched diet low or high in AGEs on the gut microbial composition of 70 abdominally obese individuals in a double-blind parallel-design randomized controlled trial (NCT03866343). Additionally, we investigated the cross-sectional associations between the habitual intake of dietary dicarbonyls, reactive precursors to AGEs, and the gut microbial composition, as assessed by 16S rRNA amplicon-based sequencing.

Results: Despite a marked percentage difference in AGE intake, we observed no differences in microbial richness and the general community structure. Only the *Anaerostipes* spp. had a relative abundance > 0.5% and showed differential abundance (0.5 versus 1.11%; p = 0.028, after low or high AGE diet, respectively). While the habitual intake of dicarbonyls was not associated with microbial richness or a general community structure, the intake of 3-deoxyglucosone was especially associated with an abundance of several genera.

Conclusions: A 4-week diet low or high in AGEs has a limited impact on the gut microbial composition of abdominally obese humans, paralleling its previously observed limited biological consequences. The effects of dietary dicarbonyls on the gut microbiota composition deserve further investigation.

Introduction

Dietary advanced glycation endproducts (AGEs), a heterogeneous group of sugar-modified proteins, are abundantly present in heated foods[1]. A diet high in AGEs has been linked to negative health outcomes, such as weight gain[2], increased risk of several cancer types[3–6], and insulin resistance, although results for the latter are inconsistent[7,8]. However, it is currently not understood how dietary AGEs elicit biological effects. Potentially, this could be mediated by modulation of the gut microbiota composition. Foods and dietary patterns are well known to be able to influence gut microbiota[9], which in turn are increasingly recognized as key players in the development of obesity[10] and cancer[11]. Although the metabolism of (individual) dietary AGEs is still largely unknown, early estimations using ELISA techniques suggest that only 10% of dietary AGEs are absorbed into circulation, so that the majority should pass through the colon[12]. Here, dietary AGEs have the potential to act as substrates for gut microbes, ultimately altering their composition. Indeed, several in vitro studies report that AGEs may be selectively metabolized by certain gut microbes[13,14]. In line with this, a baked chow diet high in AGEs has been shown to alter gut microbial composition in mice[15–17] and to reduce caecal short chain fatty acid concentrations[15,18]. However, results concerning specific genera are not consistent. Extrapolating these findings to humans is difficult, not only because of inter-species differences in gut microbiota composition but also because baking may decrease the nutritive value of proteins and micronutrients, but may also improve antioxidant capabilities of food[19].

So far, only two randomized controlled trials (RCTs) have addressed the effects of a low or high AGE diet on gut microbiota content in humans, and both were limited by their small sample size and highly selective patient groups[20,21]. In 20 male adolescents with a mean age of 12 years, comparisons of a two-week diet high and low in AGEs led to a reduction in *lactobacilli* and an expansion in *enterobacteria*[20]. In 20 peritoneal dialysis patients, a one-month diet low in AGEs compared to a habitual diet high in AGEs led to a reduction in *Prevotella copri* and *Bifidobacterium animalis*, while there was an expansion of *Alistipes indistinctus, Clostridium hatewayi, Clostridium citroniae*, and *Ruminococcus gauvreauii*[21]. However, low and high AGE diets in these trials were achieved by modulating food preparation methods through heat, which may lead to similar limitations as described above. Furthermore, these findings cannot be extrapolated to the general population. Of note, it was observed in the microbiota development that increased intake of fructoselysine via formula milk in early life is associated with increased levels of *Intestinimonas*-like bacteria that are known to convert this Amadori product into butyrate[22,23].

The consequences of dietary dicarbonyls on gut microbiota also deserve further investigation. These small and highly reactive molecules, also present in foods[24], may lead to rapid formation of AGEs within the body. Despite their high reactivity, simulated gastrointestinal digestion experiments suggest that dietary dicarbonyls may pass through the stomach and reach the colon largely unaltered[25], where they may also exert effects on the gut microbiota. Manuka honey, highest in the dicarbonyl methylglyoxal of all measured food items[26], shows strong antibacterial properties[27,28]. However, relationships between gut microbial composition and dicarbonyls from the habitual diet have not yet been investigated, as an extensive dietary dicarbonyls database has only recently been developed[26].

As such, we investigated the effects of a specifically designed 4-week diet low or high in AGEs on the gut microbiota composition of abdominally obese but otherwise healthy individuals in a parallel-design double blind RCT. Secondly, we also investigated cross-sectional associations between the habitual intake of dicarbonyls and the gut micro-biota composition in these individuals prior to the dietary intervention.

Materials and Methods

Study approval

This study was approved by the Maastricht University Medical Center ethics committee, performed in accordance with the Declaration of Helsinki, and registered at both international and national trial registries (clinicaltrials.gov: NCT03866343, trialregister.nl: NTR7594). All participants provided written informed consent.

Study population and design

A total of 82 abdominally obese but otherwise healthy individuals were recruited, as described in detail elsewhere[8]. These participants were randomly assigned to a 4-week dietary intervention low or high in AGEs at a 1:1 ratio in a double blind, parallel design. Randomization was stratified for age (below and above 50 years of age) and sex, in block sizes of 4, and performed by an independent investigator. Both the investigators and participants were blinded to the treatment allocation, and participants were instructed not to inform the investigators about the food items in their dietary intervention. Only the study dietician was aware of the treatment allocation.

Sample size calculation

This study was powered to detect a difference in the primary outcome of insulin sen-sitivity, as described elsewhere[8]. Based on this, 36 individuals per group were needed to detect a statistical difference. Considering a drop-out rate of 12%, we included 41 participants per group, resulting in a total of 82 participants.

Run-in diet

Prior to the baseline measurement, all participants followed an isocaloric two-day run-in diet. A participants' habitual energy intake was assessed by a three-day food diary. The run-in diet contained an average amount of dietary AGEs, based on intake in a large population-based cohort[29], and was designed to exclude any influences of high AGE products consumed in the days prior to the baseline measurement.

Dietary intervention

Intervention diets were constructed by a trained dietician and were energy- and macronutrient-matched. Both intervention diets adhered to the Dutch dietary guidelines for macro- and micronutrient intake[30] and contained 15 Energy % in calories (En%) protein, 35 En% fat, 48 En% carbohydrates, and 2 En% fibers. With the use of our gold standard UPLC-MS/MS dietary AGE database that contains approximately 250 food items[1], a theoretical difference of approximately 75% in AGEs was attained between diets while not solely relying on different food preparation methods. Participants prepared their food at home using predefined recurring weekly menus with extensive instructions. Most food items were provided to the participants free of charge by means of a delivery service. Participants were instructed not to change their habitual portion sizes, number of in-between snacks, and the habitual timing of their food intake, not to attempt changes in body weight, and not to consume food supplements during the duration of the study.

Dietary intake

Adherence to the dietary intervention was measured in three ways. First, participants kept a five-day dietary record in the first and last week of the intervention. Second, participants were additionally contacted in the second and third week of the intervention to assess food intake in a standardized way by a 24-h dietary recall[31]. Nutrient intake from these dietary records and recalls were determined using a nutrient software program (Compl-eat, Human Nutrition Wageningen University,

Wageningen, The Netherlands). Third, free AGEs in plasma and 24-h urine samples were compared between groups after the intervention, as described elsewhere[8].

Habitual dietary AGE and dicarbonyl intake was determined by coupling a validated semi-quantative FFQ food frequency questionnaire[32], with a reference period of one year, to our dietary AGE[8] and dicarbonyls[26] database, as described elsewhere[33].

Collection of stool samples

Participants were instructed to deposit their stool in a disposable Fecotainer ® (AT Medical B.V., Amsterdam, The Nederlands) within 24-h before their appointment to the lab. These samples were then manually homogenized by a researcher, aliquoted, and frozen at −80 °C until further analysis.

DNA isolation

DNA was extracted from 200 mg of frozen aliquots of homogenized feces by Repeated Bead Beating (RBB) combined with column-based purification according to the recommended protocol Q of the International Human Microbiome Standards Consortium[34]. Briefly, bead beating was performed using the FastPrep™ Instrument (MP Biomedicals, Santa Ana (CA), USA) with 0.1 mm zirconium-silica beads (BioSpec Products, Bartlesville (OK), USA) to homogenize feces. The DNA was finally purified by adapting it to QIAamp DNA Stool Mini kit columns (Qiagen, Hilden, Germany).

Microbiota profiling

The V4 region of the 16S rRNA gene was PCR amplified from each DNA sample using the 515F/806R primer pair described previously[35]. After 25 cycles of PCR amplification, amplicons were purified using AMPure XP purification (Agencourt, Beck-mann-Coulter, CA, USA) according to the manufacturer's instructions and eluted in 20 µL 1 × low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Quantification of amplicons was subsequently performed by the Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, Thermofisher, MA, USA) using a Victor3 Multilabel Counter (Perkin Elmer, Waltham, MA, USA). Amplicons were mixed in equimolar concentrations to ensure equal representation of each sample and sequenced on an Illumina MiSeq instrument (Illumina, Eindhoven, The Netherlands) (MiSeq Reagent Kit v3, 2 × 250 cycles, 10% PhiX) to generate paired-end reads of 250 bases in length in both directions.

The pre-processing of sequencing data, using an in-house pipeline based upon DADA2 (R version 4.1.0, R-Project)[36], consisted of the following steps: reads filtering, identification of sequencing errors, dereplication, inference, and removal of chimeric sequences. The length of the raw reads was detected using HTSeqGenie[37], and sequence manipulation was performed using Biostrings[38]. In order to assign taxonomy, DADA2 was used to annotate down to the species level using the database SILVA 138 version 2[39]. Data were expressed as Amplicon Sequence Variants (ASVs). Decontam was used with the either setting, which combines the two statistical methods' prevalence and frequency for the identification of contaminating ASVs[40]. Contaminating ASVs identified by decontam were filtered out together with ASVs presented in less than 5% of all samples and ASVs with a total abundance below 0.01% across all samples. Finally, we omitted samples with a low sequencing depth (<40000 sequences). The final file was saved in the phyloseq format from which the taxa tables were extracted[41].

Gut microbiota composition

All gut microbial analyses were performed using the R package "microViz"[42], and species with a prevalence <5% were excluded.

Alpha diversity, a measure of within-sample bacterial diversity, was expressed as both total diversity (richness) and the Shannon index. Total diversity was defined as the total number of individual ASVs within a sample. The Shannon index, a measure of species diversity, takes into account both species abundance and evenness.

Beta diversity, a measure of differences in between-sample bacterial composition, was expressed as both the Aitchison distance and the Bray–Curtis dissimilarity. These beta diversity measures were plotted using principal coordinate analysis (PCoA, for Bray–Curtis dissimilarity) and principal component analysis (PCA, for Aitchison distance) to visualize differences in the bacterial composition between groups before and after the dietary intervention. The Aitchison distance was specifically used to visualize the taxa driving differences in the microbial community structure between groups. Additionally, Bray–Curtis beta diversity was also determined as the within-subject temporal change in the microbial composition due to the low or high AGE diet. For beta diversity measures, taxa were aggregated at the genera level.

Statistics

Analyses regarding participant characteristics and dietary intake were conducted

using SPSS version 25 for Windows (IBM Corporation, Armonk, NY, USA). Analyses regarding the gut microbiota were conducted using the R package "microViz"[42]. Participant characteristics and dietary intake are presented as means ± SD, medians [interquartile range], or percentages, as appropriate. Differences in dietary intake between both groups were assessed by a one-factor ANCOVA while adjusting for sex, age, and energy intake. Differences in alpha diversity between both groups were assessed by a one-factor ANCOVA while adjusting for sex, age, and alpha diversity at baseline. Differences in overall gut microbial composition (i.e., centroid of both groups' beta diversity in the PCoA and PCA plots) were statistically tested with permutational multivariate analyses of variance (PERMANOVA). Differences in the specific gut microbial composition after the intervention (i.e., differential abundance analysis) were assessed using beta binomial regression aggregated at the genus level, adjusted for age and sex. This was also performed at baseline to rule out differences already present before the intervention. To account for multiple testing, we applied the Benjamini–Hochberg (B–H) multiple comparison correction with a false discovery rate of 10%. Results of beta binomial regression, being fold-changes between intervention diets, were visualized in a three-way taxonomic association. Subsequently, genera that were differentially abundant after the low versus the high AGE diet were transformed to relative abundance and visualized as box plots. To investigate associations between the habitual intake of dicarbonyls and measures of alpha diversity, we used multiple linear regression, adjusting for potential confounders in two separate models. In model 1, we adjusted for age and sex. In model 2, we adjusted for energy intake and the Dutch Healthy Diet score[43], in addition to age and sex. To investigate associations between the habitual intake of dicarbonyls and taxonomic abundance, we used beta binomial regression while adjusting for age, sex, energy intake, and the Dutch Healthy Diet Index. A p-value of < 0.05 was considered statistically significant.

Results

Baseline characteristics

Of 82 enrolled participants, 34 participants allocated to the low AGE diet and 36 participants allocated to the high AGE diet collected stool samples during both their baseline and follow-up visit. Reasons for missing data were mainly the inability to collect stool within the 24-h timeframe before their lab visit (n = 4) or a dropout from the intervention (n = 10) (Figure 7.1). Dropouts occurred due to reasons unrelated to the dietary intervention. By design, participants were abdominally obese but otherwise healthy (Table 7.1). The *Firmicutes/Bacteriodetes* ratio of all participants is shown in Supplementary Figure 7.1.

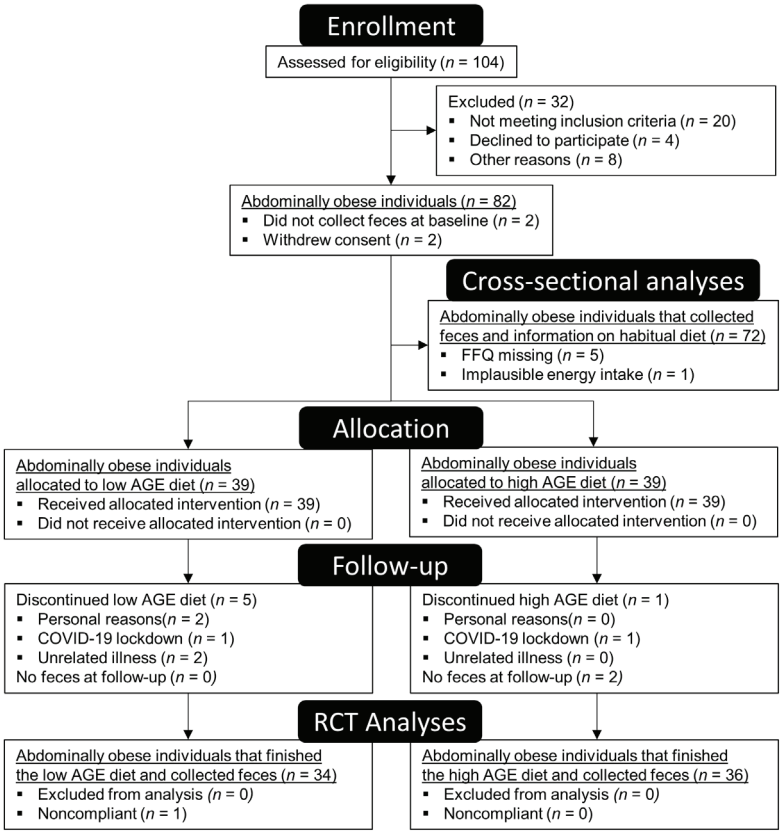

Figure 7.1. CONSORT flow diagram for RCT and cross-sectional analyses.

Table 7.1. Characteristics of 70 abdominally obese individuals included in the deAGEing trial at baseline.

Characteristic	Low AGE *n* = 34	High AGE *n* = 36
Demographics		
Age (years)	52 ± 13	54 ± 13
Males/Females	10/24	11/25
Weight (kg)	87.7 ± 14.3	88.0 ± 13.1
Waist circumference (cm)		
Men	106.7 ± 4.8	107.5 ± 7.1
Women	101.2 ± 8.6	100.1 ± 8.2
BMI (kg/m^2)	30.4 ± 4.1	30.8 ± 4.2
24-h systolic BP (mmHg) [1]	126 ± 13	124 ± 9
24-h diastolic BP (mmHg) [1]	80 ± 9	77 ± 7
Biological		
Fasting glucose (mmol/L)	4.9 ± 0.4	5.1 ± 0.5
Total cholesterol (mmol/L)	5.0 ± 0.9	5.4 ± 0.8
LDL cholesterol (mmol/L)	3.3 ± 0.9	3.7 ± 0.7
HDL cholesterol (mmol/L)	1.4 ± 0.4	1.3 ± 0.3
Triglycerides (mmol/L)	1.2 ± 0.4	1.6 ± 0.7
Feces		
Richness (observed species)	194 ± 30	173 ± 32
Shannon index	4.08 ± 0.27	3.93 ± 0.30
Bristol stool scale	4 ± 1	4 ± 1

Data are presented as means ± SD. [1] Low AGE *n* = 32, High AGE *n* = 35.

Dietary intake during the intervention

As published previously[8], the intake of dietary AGEs during the 4-week intervention period was increased 2.5–5.2 fold in the high AGE group in comparison to the low AGE group, and this difference was confirmed by significantly higher levels of free AGEs in the plasma and urine after the high AGE diet compared to the low AGE diet. (Table 7.2). By comparison, the habitual intake of AGEs in this cohort, assessed by a FFQ, was 4.07 ± 1.71 mg/day for CML, 3.83 ± 1.78 mg/day for CEL, and 26.98 ± 10.22 mg/day for MG-H1. Importantly, daily energy intake during the intervention was not statistically different between groups, and there was no difference in body weight after the intervention (mean difference (kg) [95% CI] for a low vs. high AGE diet of −0.4 [−1.09,0.3], p = 0.31). Although the intervention diets were by design matched for macronutrients, the actual intake of energy as fat and carbohydrates was marginally but statistically different between groups (Table 7.2). One participant, allocated to the low AGE diet was deemed non-compliant based on a large increase in free AGEs in their plasma (37% for CML, 197% for CEL, and 568% for MG-H1)[8]. However, as per the intention-to-treat design, this participant was included in all analyses. Despite this, we performed sensitivity analyses for all intervention-related outcomes excluding this participant. In the case of different results, this was noted in the respective sections.

Table 7.2. Average daily AGE, dicarbonyl, energy, and macronutrient intake of 70 abdominally obese individuals during the low or high AGE dietary intervention.

Nutrient	Low AGE n = 32[1]	High AGE n = 36	Low vs. High p
AGEs (mg/day)			
CML	2.68 ± 0.67	6.90 ± 1.32	<0.001
CEL	1.72 ± 0.40	8.94 ± 1.98	<0.001
MG-H1	13.67 ± 3.11	48.75 ± 11.93	<0.001
Dicarbonyls (mg/day)			
MGO	3.04 ± 0.89	3.76 ± 1.00	<0.001
GO	2.84 ± 0.73	3.20 ± 0.70	<0.001
3-DG	13.86 ± 5.33	19.15 ± 5.88	<0.001
Energy (kcal/day)			
Energy intake[2]	2034 ± 476	2078 ± 471	0.612
Macronutrients (energy %)			
Protein	17.1 ± 1.6	16.7 ± 1.5	0.325
Plant-based protein	6.4 ± 0.8	7.6 ± 0.6	<0.001
Animal-based protein	10.7 ± 1.8	9.1 ± 1.6	<0.001
Fat	31.6 ± 2.6	35.6 ± 3.0	<0.001
Saturated fat	12.8 ± 1.5	12.0 ± 0.8	0.009
Mono-unsaturated fat	9.7 ± 0.8	12.7 ± 1.6	<0.001
Poly-unsaturated fat	6.1 ± 1.1	7.7 ± 1.5	<0.001
Carbohydrates	48.4 ± 2.7	44.7 ± 2.8	<0.001
Mono- and disaccharides	21.2 ± 2.8	19.4 ± 2.7	0.008
Polysaccharides	27.2 ± 2.3	25.3 ± 1.5	<0.001
Fiber	2.1 ± 0.2	2.3 ± 0.1	0.001
Alcohol	0.0 [0.0,0.60]	0.0 [0.0,0.76]	0.966

Daily intakes (means ± SD, medians [IQR]) were assessed from two five-day dietary logs in week one and week four of the intervention. Differences between intervention groups were tested by a one-factor ANCOVA with energy intake, sex, and age as covariates, and differences in alcohol intake were tested by the non-parametric Mann–Whitney U test. [1] Dietary logs were not returned by two participants in the low AGE group. [2] Energy intake was not included as a covariate.

Microbial richness and diversity following the low and high AGE diet

First, we determined the effect of the low versus the high AGE diet on the observed microbial richness and diversity, expressed as the Shannon index. Although a trend of decreased microbial richness after the low AGE diet was observed, no differences after the low versus the high AGE diet were found (Figure 7.2). Likewise, there was no difference in microbial diversity (Shannon index) after the low versus the high AGE diet (Figure 7.2).

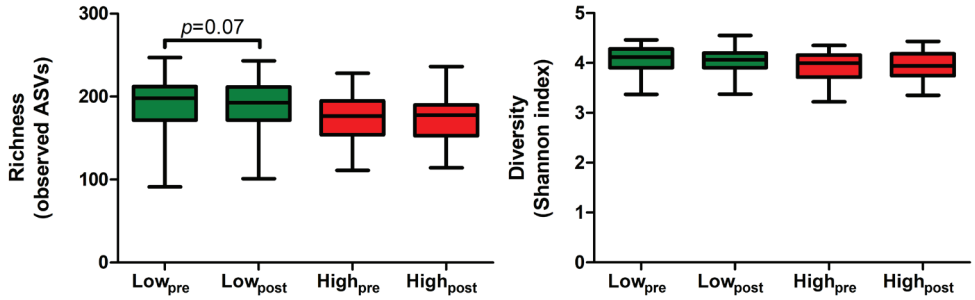

Figure 7.2. Richness (left) and gut microbial diversity (right) before and after a 4-week diet low or high in AGEs. Sample sizes: low AGE group *n* = 34, high AGE group *n* = 36. Within-group differences were tested with a paired samples t-test. Treatment effects were tested with a one-way ANCOVA with adjustment for age, sex, and the baseline variable of interest.

Microbial community structure following the low and high AGE diet

Next, we investigated changes in the general community structure expressed as either the Bray–Curtis dissimilarity or the Aitchison distance. Based on PCoA plots of Bray–Curtis dissimilarity, we observed no apparent difference in the overall microbial composition after the low versus the high AGE diet (Figure 7.3, upper row). PERMANOVA analysis revealed a borderline significant difference in centroids between groups (p = 0.078), which was less apparent at baseline (p = 0.341). To identify other potential diet-induced changes in the overall microbial composition, we next tested whether the intra-individual change in the Bray–Curtis dissimilarity and the overall Bray–Curtis dissimilarity of all participants within a group were different after the low versus the high AGE diet. However, there was no difference in the intra-individual change in the Bray–Curtis dissimilarity due to the low or high AGE diet (Supplementary Figure 7.2). Although the within-group beta diversity was statistically significantly lower after the low versus the high AGE diet, indicating a more similar microbial composition, the overall difference between groups was only −0.01 [−0.02,−0.00] (Supplementary Figure 7.2).

We also visualized the general community structure with PCA plots of the Aitchison dissimilarity, showing taxa underlying the ordination. This revealed that the taxa driving a potential difference in the general community structure after both diets (including *Oscillispiraceae Family, Prevotella*, and *Holdemanella*) already tended to do so before allocation of the dietary intervention. (Figure 7.3, lower row).

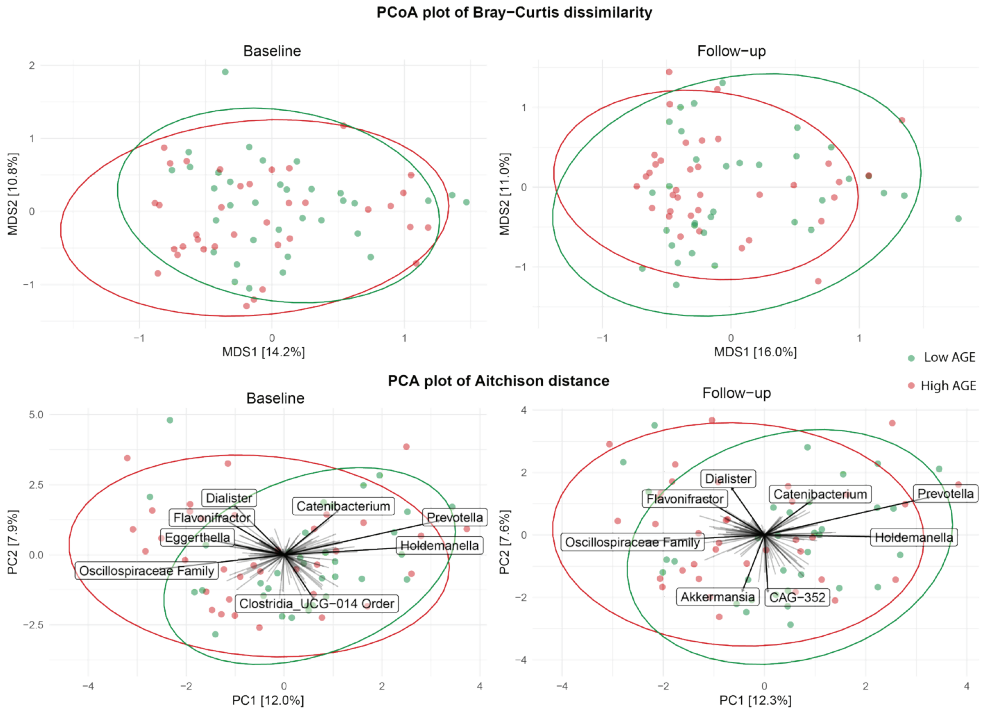

Figure 7.3. Measures of beta diversity before (left column) and after (right column) a low or high AGE diet in abdominally obese individuals. Upper row: principle coordinate analysis of Bray–Curtis dissimilarity. Lower row: Principle component analysis of the Aitchison distance. Sample sizes: low AGE group *n* = 34, high AGE group *n* = 36.

Differentially abundant genera after the low and high AGE diet

Next, we determined specific changes in the gut microbial composition by comparing the relative abundance of all genera after the low versus the high AGE diet using beta-binomial regression. After adjusting for sex and age, and disregarding differentially abundant genera at the baseline, the low versus the high AGE diet led to an enrichment in the genera *Tyzzerella* and *Family_XII_UCG-001*, and to a contraction in the genera *Negativibaccillus, Oscillibacter*, and *Anaerostipes* (Supplementary Figure 7.3). Of these differentially abundant genera, *Anaerostipes* had the highest median relative abundance (0.55% [0.31,1.29] vs. 1.11% [0.67,2.05] after the low versus the high AGE diet) (Figure 7.4). Furthermore, these comparisons lost statistical significance

after correction for multiple testing (all *p* values > B-H critical *q* of 0.004). Of note, after exclusion of the non-compliant participant, there was an enrichment in the genus *Christensenellaceae_R-7 Group* after the low versus the high AGE diet (median relative abundance (%) [95% CI] of 0.70% [0.40,2.25] vs. 0.62% [0.28,1.27]), while all other comparisons were materially unchanged (Figure 7.4).

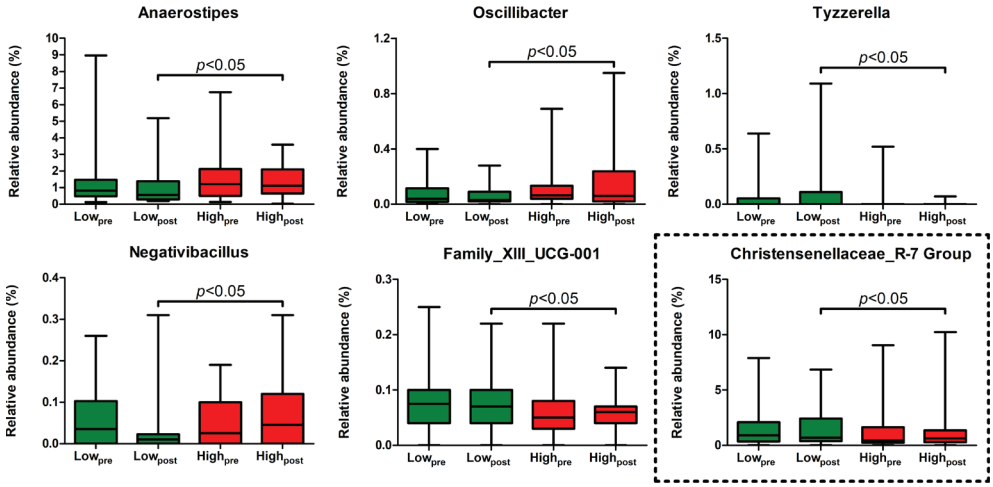

Figure 7.4. Relative abundance of differentially abundant genera after a 4-week low (in green) or high AGE diet (in red) in abdominally obese individuals. *n* = 34 for the low AGE group and *n* = 36 for the high AGE group. Statistical significance was assessed using beta binomial regression with adjustments for age and sex. Please note that the difference in the relative abundance of the *Christensenellaceae_R-7* Group only became statistically significant after exclusion of a non-compliant participant of the low AGE group. This participant was included in all other comparisons. All comparisons became statistically non-significant after correction for multiple testing.

Associations between habitual intake of dicarbonyls and gut microbial composition

Finally, we also investigated associations between the habitual intake of dicarbonyls, assessed before the allocation to the low and high AGE interventions, and alpha diversity, beta diversity, and microbial abundance. The mean habitual intake of dicarbonyls was 3.74 ± 1.49 mg/day for MGO and 3.59 ± 1.42 mg/day for GO, and the median for 3-DG was 14.54 [10.37,24.81] mg/day. There was a trend of decreased microbial diversity (Shannon index) with a higher intake of 3-DG (standardized beta (95% CI) of −0.07 [−0.14,0.01] after adjusting for age and sex. Although additionally adjusting for energy intake and the Dutch Healthy Diet Index further widened the confidence interval, the effect size remained similar (−0.07 [−0.18,0.03]) (Supplementary Table 7.1). Greater intake of 3-DG was not associated with microbial richness. In line with this, the habitual intake of dietary MGO and GO were also not associated with microbial richness or diversity (Shannon index) (Supplementary

Table 7.1). Likewise, PERMANOVA analysis revealed that the habitual intake of these dicarbonyls did not predict variation in the Bray–Curtis dissimilarity (p = 0.54 for MGO, p = 0.64 for GO, and p = 0.60 for 3-DG, data not shown).

In contrast, beta-binomial regression revealed several associations between the habitual intake of 3-DG and genera abundance. After adjusting for age, sex, energy intake, and the Dutch Healthy Diet index, and with correction for multiple testing, a higher habitual intake of 3-DG was positively associated with *Anaerostipes, Fusicatenibacter*, and *Tyzzerella*, and inversely associated with the *Adlercreutzia, Family_XIII_AD3011 group* and the *Eubacterium Siraeum group* (p < B-H critical q of 0.005) (Supplementary Figure 7.4). A higher habitual intake of GO was inversely associated with *Colidextribacter, Gastranaerophilales order*, the *Ruminoccocus gnavus group*, and Veilonella (p < B-H critical q of 0.003) (Supplementary Figure 7.5). After correction for multiple testing, the habitual intake of MGO was not associated with genera abundance (all p > B-H critical q of 0.005) (Supplementary Figure 7.6). Relative abundances of these genera are shown in Supplementary Table 7.2. Interestingly, the observed associations were not shared between dietary dicarbonyls.

Discussion

In the present double-blind parallel-design RCT, we show limited effects of a specifically designed 4-week diet low or high in AGEs on the gut microbial composition of 70 abdominally obese individuals, despite a large difference in AGE intake between the groups. In contrast, the habitual intake of dicarbonyls, reactive precursors of AGEs, was associated with both lower and higher abundances of several genera.

The intake of AGEs in our low and high AGE diet, based on regular food items and not solely on different preparation methods, was markedly different (157% for CML, 420% for CEL, and 257% for MG-H1). Despite this, energy intake was similar between groups, and there were no large differences in macronutrient intake. Compliance with the intervention was further confirmed by the increase in AGEs in plasma and urine after the high- compared to the low AGE diet[8]. Several meta-analyses suggest that a diet high in AGEs is linked to negative biological effects[44-46]. The biological mechanisms underlying dietary AGE-induced effects are largely unknown since mechanisms occurring with the endogenous formation of AGEs cannot be directly extrapolated. From the observations that most dietary AGEs reach the colon undigested[12] and that diet is a key modulator of the gut microbial composition[9], an interplay between dietary AGEs and gut microbes has been proposed as a contributing mechanism to the harmful effects of dietary AGEs. Indeed, modulation of the gut microbial composition of mice after a baked chow diet high in AGEs has been shown[15–17,20].

Despite these findings in mice, we observed no differences in microbial diversity and richness (alpha diversity) or overall gut microbial composition (beta diversity) after the low versus the high AGE diet. In agreement with our findings, Yacoub et al. showed no difference in the Shannon–Wiener index after a one-month diet low or habitual with regard to dietary AGEs in 20 peritoneal dialysis patients[21]. In contrast, they showed a separation in the gut microbiota composition after both diets using dimension reduction analysis, although this is not directly comparable to our measures of beta diversity. In line with our unchanged alpha and beta diversities, our low AGE diet compared to the high AGE diet only led to changes in the abundance of five genera with a low relative abundance. None of these genera were changed in the two other human trials[20,21].

In the main analyses of the deAGEing trial, we observed no effects of diets low or high in AGEs on insulin sensitivity, clearance, and secretion, vascular function, overall inflammation, or biochemical parameters[8]. Our findings, therefore, not only suggest limited consequences of a 4-week diet low or high in AGEs on the gut microbial composition, but also of limited biological effects overall. As such, our results are in apparent disagreement with the two aforementioned trials in humans[20,21], but also with trials in mice[15–17,47,48]. Differences in the study design could explain the discrepancies between our data and both the human and mice trials. Most importantly, the external validity of these findings is limited, as there are large inter-species differences in the gut microbiota composition between mice and humans, but also within the study populations of the human trials (i.e., abdominally obese but healthy adults vs. adolescent boys[20] and peritoneal dialysis patients[21]). Unfortunately, a direct comparison in AGE intake between these studies is not possible, as AGEs in food were not determined by the gold standard of mass spectrometry in these other studies. In addition, it is unclear whether the results of previous studies can solely be ascribed to dietary AGEs. The modulation of dietary AGEs in these studies was induced by differences in cooking methods, which may also lead to the formation of other Maillard reaction products such as acrylamide[49], a carcinogenic compound in mice[50], or lead to the degradation of heat-sensitive vitamins. To avoid such limitations, we have used a specifically designed dietary intervention based on regular food items in our gold standard dietary AGE database. Furthermore, we used sophisticated statistical methods for our differential abundance analysis. “Standard” non-parametric statistical tests, such as the Mann–Whitney U or the Wilcoxon rank-sum test, do not appropriately take into account the compositional nature of the data and are subject to inflated false discovery rates[51-53]. However, a limitation of our trial, compared to the animal studies, is that a 4-week diet may be too short to result in changes in the gut microbial composition. Other general limitations include the analysis of 16S rRNA instead of metagenomics sequencing and the focus on markers based on relative abundance instead of absolute abundance.

The small changes in genera abundance after the low versus the high AGE diet in the present study are unlikely to have large health implications. Additionally, all

comparisons became statistically non-significant after adjusting for multiple testing. However, if anything, the low versus the high AGE diet may have led to a less favorable microbiota profile. Most notably, we observed a decreased abundance of *Anaerostipes* after the low versus the high AGE diet. Although we hypothesized that a low AGE diet would improve insulin sensitivity and therefore decrease the risk of future diabetes, a decreased abundance of *Anaerostipes* is suggestive of a more diabetes-prone phenotype. *Anaerostipes* is among the 15 most abundant taxa in healthy individuals[54], and it may use inulin and fructo-oligosaccharides, present in many foods including onions, via trophic chains with the *Bifidobacterium* spp.[55] or even in pure culture (Dr. Nam Bui, unpublished observations) to produce butyrate, a beneficial SCFA that contributes to insulin sensitivity in animal studies[56]. Moreover, a recently discovered propionate-production gene cluster of the *Anaerostipes* species has been associated with beneficial metabolic biomarkers in (pre)diabetes cohorts[57]. One potential explanation for the decreased abundance of the *Anaerostipes* spp. in the low AGE group may relate to the level of fiber intake, which was slightly albeit statistically significantly higher in the high AGE group compared to the low AGE group. Moreover, it is unknown whether the nature of fiber intake was different between these groups as these data were not available. Other potential unfavorable shifts in genera abundance were those of *Tyzerella* and *Family_XII_UCG-001*, which both increased in abundance after the low versus the high AGE diet. These genera were enriched in patients with general anxiety disorder[58] and irritable bowel syndrome[59], and ulcerative colitis[60], respectively. In contrast, *Oscillibacter*, which decreased in abundance after the low versus the high AGE diet, was enriched in patients with chronic kidney disease and showed positive correlations with uremic metabolites[61]. Of note, a recently discovered *Oscillibacter*-related species, *Dysosmobacter welbionis*, was found to improve insulin sensitivity in mice[62]. *Negativibacillus* has only recently been isolated in humans[63] and has, to our knowledge, not been associated with disease states. Although we observed an increased abundance of the *Christensenellaceae_R-7* Group after the low versus the high AGE diet, and genera of this family are inversely associated with adiposity in humans[64], this comparison only reached statistical significance after exclusion of a noncompliant participant, and its relevance should therefore be interpreted with caution. All in all, it is possible that any beneficial effects of the low AGE diet on insulin sensitivity were counteracted by less favorable effects on the gut microbiota, ultimately leading to no change in insulin sensitivity after the low versus the high AGE diet.

Research on the biological effects of dietary dicarbonyls in humans is scarce, but studies so far are suggestive of beneficial effects. Recently, Maasen et al. showed an inverse association between the greater habitual intake of MGO, but not 3-DG or GO, and a sum score of low-grade inflammation plasma biomarkers in the population-based cohort of the Maastricht Study [manuscript submitted]. How dietary dicarbonyls could exert biological effects is incompletely understood, but options

include direct uptake into the circulation[65], endogenous formation of new AGEs, or an effect on the gut microbiota. Regarding the latter, we showed no association between habitual dietary dicarbonyl intake and alpha or beta diversity, but there was a trend of decreased gut microbial richness with the higher intake of 3-DG. This is in agreement with a recent fluorescence in situ hybridization (FISH) analysis showing that of all three dicarbonyls, the antimicrobial capacity of 3-DG was highest[25]. In contrast, a greater habitual intake of 3-DG was associated with a higher abundance of two beneficial genera. Specifically, after adjusting for multiple testing, a higher habitual intake of 3-DG was associated with a higher abundance of *Anaerostipes* and *Fusicatenibacter* (median relative abundance % [IQR] of 1.3% [0.8,2.6]). This genus' only known species, *Fusicatenibacter saccharivorans*, was reported to be positively associated with production of the anti-inflammatory cytokine IL-10 in ulcerative colitis patients[66]. These combined findings suggest that the beneficial associations between the habitual intake of dicarbonyls may at least partly be mediated by an effect on the gut microbiota composition. Furthermore, as the high AGE diet in the present study was also higher in dicarbonyls, albeit to a much lesser extent, we cannot exclude the possibility that the increased intake of dicarbonyls in the high AGE group influenced our results. Although we observed very limited effects of the intervention diets on our outcomes overall, there was an increase in plasma adiponectin after the high- compared to the low AGE diet[8]. Potentially, this was a result of the increased intake of dicarbonyls rather than AGEs. Interestingly, most associations between the habitual intake of dicarbonyls and genera abundance were not shared between the individual dicarbonyls, suggesting unique relationships. However, all in all, these analyses should be interpreted with caution and mainly serve as a stepping-stone for further research. Although we did adjust for several important potential confounders—age, sex, energy intake, and the Dutch Healthy Diet index—our limited sample size of 72 participants restricted adjustments for additional variables. We also did not measure short and branched chain fatty acids, which could have provided more insight into the biological relevance of these associations as well as the changes in genera abundance after the low and high AGE diets. Another limitation, especially regarding the estimations of habitual dicarbonyl intake, is that FFQs may be prone to recall bias[67], and no FFQ so far has been validated for estimating dicarbonyl intake. A final limitation is that the low and high AGE diets were not matched for their glycemic load and index. Although doing so is difficult, these factors may influence the endogenous formation of AGEs[68].

To conclude, we report limited consequences of a 4-week diet low or high in AGEs on the gut microbiota composition of abdominally obese but otherwise healthy individuals. The habitual intake of dietary dicarbonyls, especially 3-DG, showed positive and inverse associations with the abundance of several genera. The effects of dietary dicarbonyls on the gut microbiota composition should be evaluated in larger observational studies and randomized controlled trials using a well-controlled dietary intervention.

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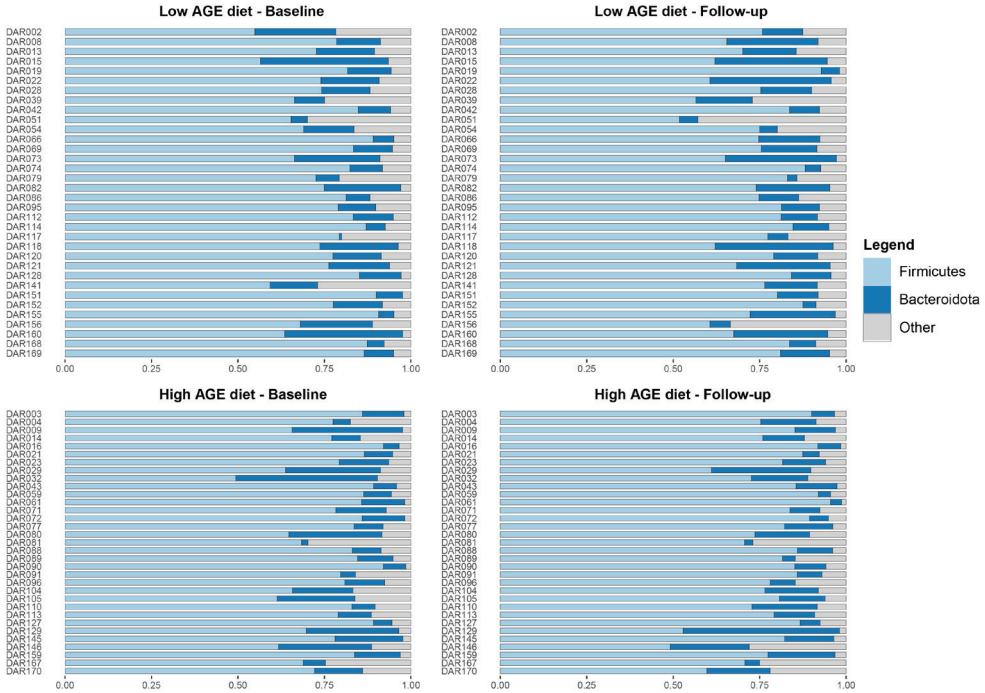
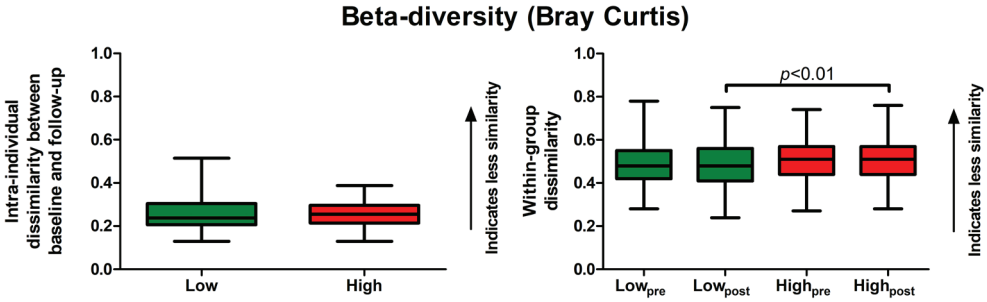
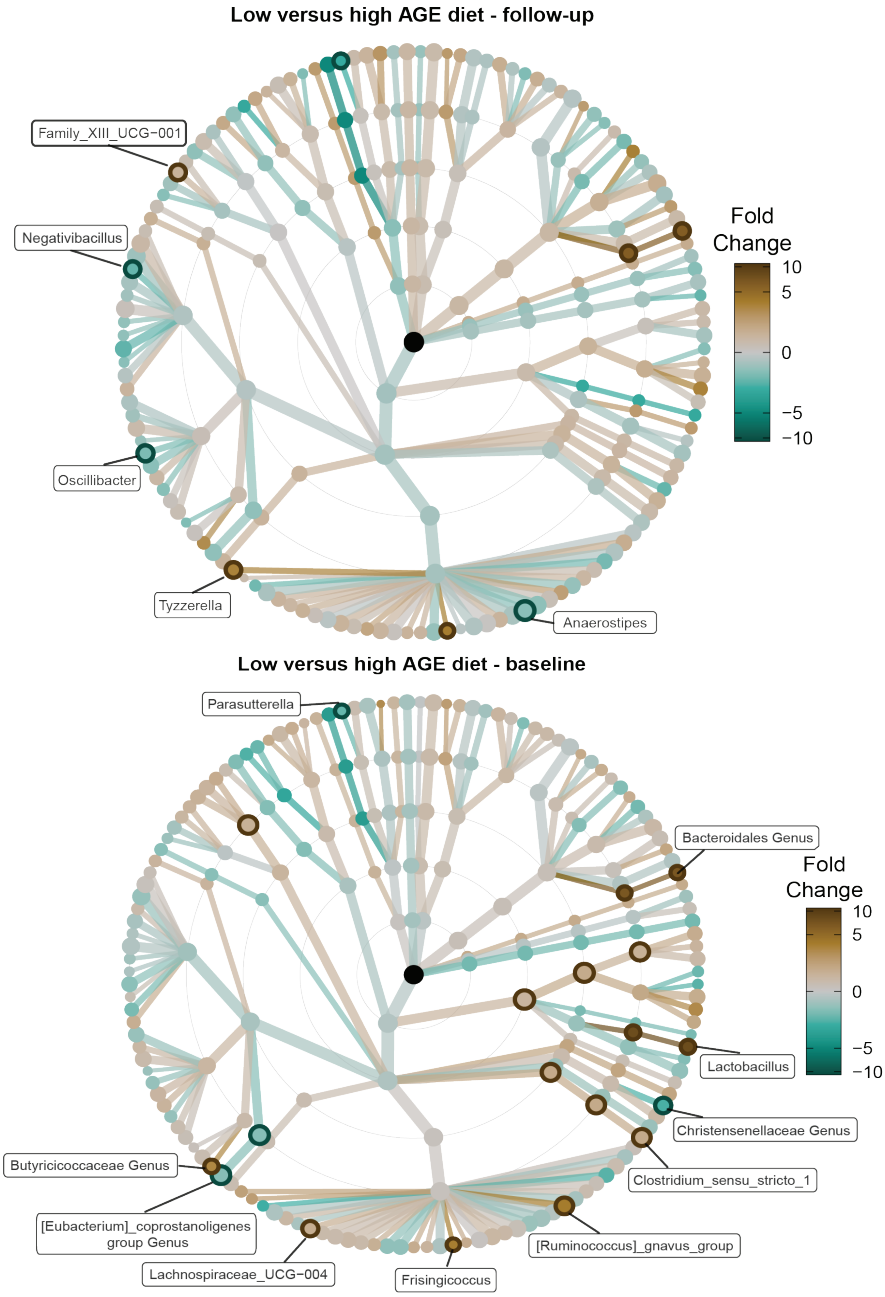
Supplementary Figures



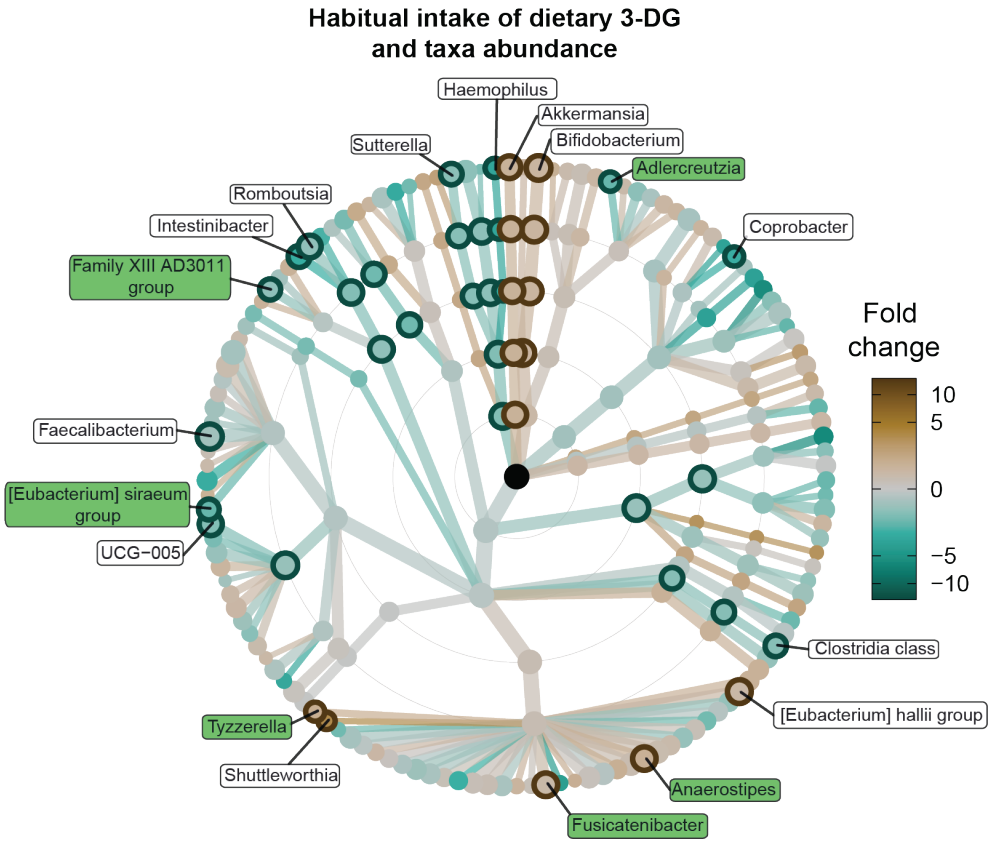
Supplementary Figure 7.1 *Firmicutes/Bacteroidota* ratio in abdominally obese individuals that finished a 4-week diet low or high in advanced glycation end products (n = 70). "DAR" indicate unique participant identifiers (Dietary AGE Restriction).



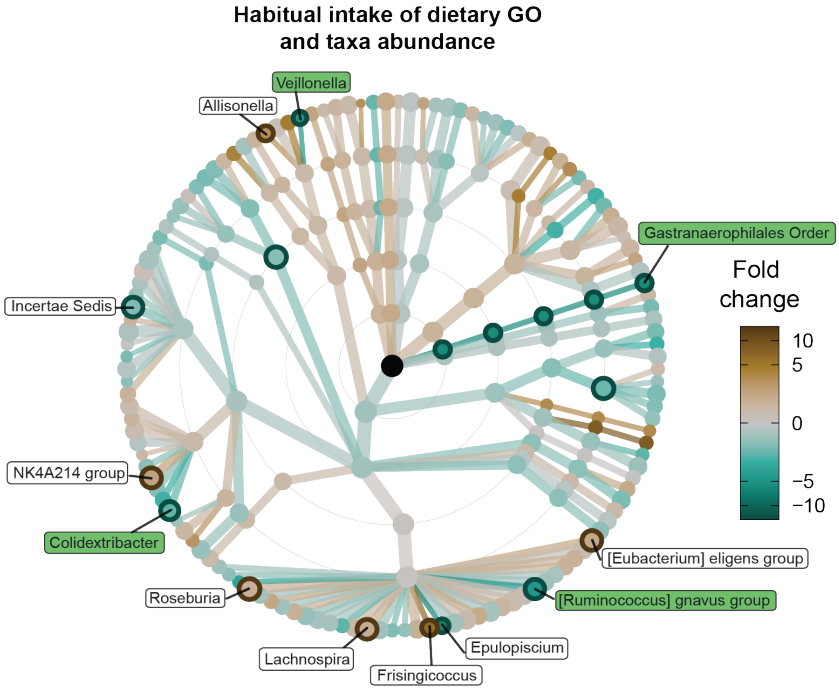
Supplementary Figure 7.2 Intra-individual (left) and within-group (right) Bray-Curtis dissimilarity after a 4-week diet low or high in advanced glycation endproducts in abdominally obese individuals (n = 34 for the low AGE diet, n = 36 for the high AGE diet).



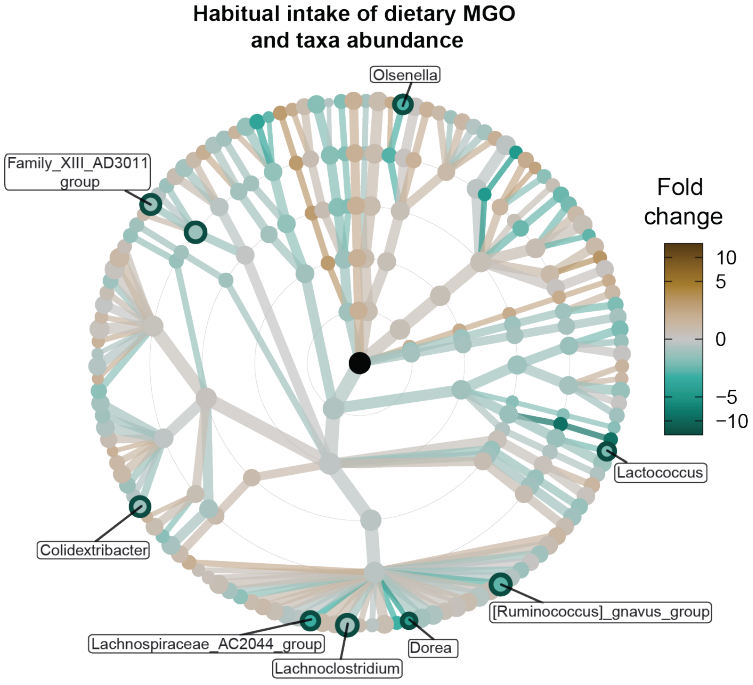
Supplementary Figure 7.3 Taxonomic association three showing differentially abundant taxa after a 4-week low (*n* = 34) compared to the high AGE (*n* = 36) diet in abdominally obese individuals (above), and before allocation to the intervention diets (below). Statistical significance was assessed using beta binomial regression with additional adjustment for age and sex. Differentially abundant taxa are shown bold and labeled (before correction for multiple testing), and marked green (after correction for multiple testing). Taxa that were already differentially abundant at baseline were not labeled in the taxonomic association three after the intervention diets.



Supplementary Figure 7.4 Associations between habitual intake of 3-deoxyglucosone (3-DG, SD/day) and taxa abundance in 72 abdominally obese individuals. Statistical significance was assessed using beta binomial regression with adjustment for age, sex, energy intake, and the Dutch Healthy Diet index. Differentially abundant taxa are shown bold and labeled (before correction for multiple testing), and marked green (after correction for multiple testing).



Supplementary Figure 7.5 and 7.6 Associations between habitual intake of glyoxal (GO, SD/day, above) and methylglyoxal (MGO, SD/day) and taxa abundance in 72 abdominally obese individuals. Statistical significance was assessed using beta binomial regression with adjustment for age, sex, energy intake, and the Dutch Healthy Diet index. Differentially abundant taxa are shown bold and labeled (before correction for multiple testing), and marked green (after correction for multiple testing).



Supplementary Tables

Supplementary Table 7.1 Associations between habitual intake of dicarbonyls and alpha diversity measures in abdominally obese individuals (*n* = 72)

	Richness (total ASVs) Beta (95% CI)	**Diversity (Shannon index)** Beta (95% CI)
Dietary MGO		
Crude model	1 (-7,9)	-0.01 (-0.08,0.06)
Model 1	-2 (-10,6)	-0.02 (-0.10,0.06)
Model 2	-3 (-16,10)	0.04 (-0.08,0.16)
Dietary GO		
Crude model	1 (-7,9)	-0.03 (-0.10,0.05)
Model 1	-2 (-10,6)	-0.03 (-0.11,0.04)
Model 2	0 (-19,19)	0.05 (-0.12,0.23)
Dietary 3-DG (ln-transformed)		
Crude model	-1 (-9,7)	-0.07 (-0.13,0.00)
Model 1	-2 (-10,6)	-0.07 (-0.14,0.01)
Model 2	-2 (-13,10)	-0.07 (-0.18,0.03)

Regression coefficients (standardized beta's and 95% CIs) represent the change in diversity or richness per 1 SD increase in daily MGO, GO, and 3-DG intake. Dietary 3-DG was log-transformed to attain a normal distribution. Abbreviations: 3-DG: 3-deoxyglucosone. GO: Glyoxal. MGO: Methylglyoxal. Model 1: Adjusted for age and sex. Model 2: Additionally adjusted for energy intake and the Dutch Healthy Diet Index.

Supplementary Table 7.2 Gut microbiota relative abundance in 72 abdominally obese individuals

Genus	**Relative abundance**		
	Median	**Quartile 1**	**Quartile 3**
Blautia	7,26%	4,59%	9,95%
Faecalibacterium	6,61%	4,36%	9,16%
Ruminococcus	5,83%	1,99%	12,71%
Agathobacter	4,88%	2,49%	9,45%
Subdoligranulum	4,13%	2,76%	7,03%
Bacteroides	3,95%	2,54%	7,57%
Bifidobacterium	2,83%	0,71%	5,02%
Coprococcus	2,59%	1,47%	4,88%
Dorea	2,11%	1,42%	3,12%
[Eubacterium]_hallii_group	1,84%	1,19%	2,48%
Roseburia	1,59%	0,94%	2,41%
[Eubacterium]_coprostanoligenes_group Family	1,40%	0,79%	2,40%
UCG-002	1,32%	0,78%	1,94%
Fusicatenibacter	1,29%	0,74%	2,21%
[Ruminococcus]_torques_group	1,27%	0,84%	2,60%
Collinsella	1,17%	0,68%	1,51%
Anaerostipes	1,08%	0,50%	1,79%
Alistipes	0,81%	0,39%	1,34%
Streptococcus	0,79%	0,33%	1,96%
Christensenellaceae_R-7_group	0,78%	0,27%	1,88%
Parabacteroides	0,76%	0,41%	1,17%
UCG-005	0,75%	0,38%	1,20%
Lachnospiraceae Family	0,64%	0,49%	1,02%
Lachnospiraceae_ND3007_group	0,62%	0,34%	1,00%
Lachnospiraceae_NK4A136_group	0,57%	0,27%	1,16%
Lachnoclostridium	0,55%	0,40%	1,03%
[Ruminococcus]_gauvreauii_group	0,51%	0,31%	0,79%
Butyricicoccus	0,49%	0,33%	0,80%
Lachnospira	0,49%	0,18%	0,80%
NK4A214_group	0,46%	0,19%	0,92%
Romboutsia	0,43%	0,14%	1,19%
[Eubacterium]_eligens_group	0,38%	0,15%	0,78%

Supplementary Table 7.2 (continued)

Genus	Relative abundance		
	Median	Quartile 1	Quartile 3
Incertae_Sedis	0,37%	0,20%	0,82%
Clostridia_UCG-014 Order	0,36%	0,02%	1,31%
Erysipelotrichaceae_UCG-003	0,33%	0,08%	0,87%
[Eubacterium]_ventriosum_group	0,30%	0,14%	0,46%
Monoglobus	0,30%	0,13%	0,56%
Oscillospiraceae Family	0,24%	0,00%	0,54%
Clostridium_sensu_stricto_1	0,24%	0,04%	1,02%
CAG-56	0,21%	0,02%	0,39%
Holdemanella	0,21%	0,00%	2,08%
Lachnospiraceae_FCS020_group	0,19%	0,10%	0,30%
Phascolarctobacterium	0,19%	0,00%	0,55%
Prevotella	0,18%	0,00%	5,12%
Sutterella	0,17%	0,04%	0,35%
Family_XIII_AD3011_group	0,15%	0,09%	0,27%
Colidextribacter	0,14%	0,05%	0,22%
Akkermansia	0,14%	0,00%	1,14%
Marvinbryantia	0,13%	0,07%	0,25%
Intestinimonas	0,12%	0,04%	0,30%
Lachnospiraceae_UCG-001	0,12%	0,03%	0,23%
Lachnospiraceae_UCG-004	0,10%	0,03%	0,19%
Intestinibacter	0,09%	0,01%	0,47%
Bilophila	0,08%	0,00%	0,12%
Eggerthellaceae Family	0,07%	0,00%	0,32%
Senegalimassilia	0,07%	0,00%	0,21%
[Eubacterium]_xylanophilum_group	0,07%	0,00%	0,20%
Escherichia-Shigella	0,06%	0,02%	0,61%
Family_XIII_UCG-001	0,06%	0,04%	0,09%
UCG-003	0,06%	0,01%	0,11%
Barnesiella	0,05%	0,00%	0,26%
Oscillibacter	0,05%	0,02%	0,12%
Ruminococcaceae Family	0,05%	0,01%	0,15%
Clostridia Class	0,05%	0,02%	0,19%
Dialister	0,04%	0,00%	2,00%
Lachnospiraceae_UCG-010	0,04%	0,00%	0,10%
Negativibacillus	0,03%	0,00%	0,10%
Adlercreutzia	0,03%	0,00%	0,07%
UBA1819	0,03%	0,01%	0,05%
Slackia	0,03%	0,00%	0,10%
[Eubacterium]_siraeum_group	0,03%	0,00%	0,11%
Actinomyces	0,03%	0,02%	0,05%
Methanobrevibacter	0,03%	0,00%	0,56%
Odoribacter	0,02%	0,01%	0,05%
UCG-010 Family	0,02%	0,00%	0,07%
GCA-900066575	0,02%	0,00%	0,05%
Turicibacter	0,02%	0,00%	0,09%
Enterorhabdus	0,01%	0,00%	0,23%
Christensenellaceae Family	0,01%	0,00%	0,13%
Flavonifractor	0,01%	0,00%	0,04%
DTU089	0,01%	0,00%	0,03%
Candidatus_Soleaferrea	0,01%	0,00%	0,02%
[Eubacterium]_brachy_group	0,01%	0,00%	0,03%
Coriobacteriales_Incertae_Sedis Family	0,01%	0,00%	0,08%
Erysipelatoclostridium	0,01%	0,00%	0,04%
Parasutterella	0,01%	0,00%	0,02%
Terrisporobacter	0,00%	0,00%	0,07%
Coprobacter	0,00%	0,00%	0,03%
CAG-352	0,00%	0,00%	0,09%
[Eubacterium]_ruminantium_group	0,00%	0,00%	0,15%
[Ruminococcus]_gnavus_group	0,00%	0,00%	0,04%

Supplementary Table 7.2 (continued)

Genus	Relative abundance		
	Median	Quartile 1	Quartile 3
Catenibacterium	0,00%	0,00%	0,01%
Succinivibrio	0,00%	0,00%	0,00%
Paraprevotella	0,00%	0,00%	0,36%
Solobacterium	0,00%	0,00%	0,00%
Muribaculaceae Family	0,00%	0,00%	0,00%
Alloprevotella	0,00%	0,00%	0,00%
Mogibacterium	0,00%	0,00%	0,00%
Klebsiella	0,00%	0,00%	0,00%
Megasphaera	0,00%	0,00%	0,00%
Acidaminococcus	0,00%	0,00%	0,00%
RF39 Order	0,00%	0,00%	0,08%
Prevotellaceae_NK3B31_group	0,00%	0,00%	0,00%
Haemophilus	0,00%	0,00%	0,03%
Mitsuokella	0,00%	0,00%	0,00%
Tyzzerella	0,00%	0,00%	0,00%
Desulfovibrio	0,00%	0,00%	0,25%
Lachnospiraceae_AC2044_group	0,00%	0,00%	0,01%
Megamonas	0,00%	0,00%	0,00%
Olsenella	0,00%	0,00%	0,05%
Peptococcus	0,00%	0,00%	0,00%
Butyricicoccaceae Family	0,00%	0,00%	0,00%
Bacteroidales Order	0,00%	0,00%	0,00%
Lactobacillus	0,00%	0,00%	0,00%
Rikenellaceae_RC9_gut_group	0,00%	0,00%	0,00%
Howardella	0,00%	0,00%	0,06%
Eggerthella	0,00%	0,00%	0,02%
Lactococcus	0,00%	0,00%	0,01%
Erysipelotrichaceae Family	0,00%	0,00%	0,04%
Rhodospirillales Order	0,00%	0,00%	0,03%
Gastranaerophilales Order	0,00%	0,00%	0,00%
Frisingicoccus	0,00%	0,00%	0,00%
Izemoplasmatales Order	0,00%	0,00%	0,00%
Faecalitalea	0,00%	0,00%	0,00%
Sellimonas	0,00%	0,00%	0,00%
Prevotellaceae Family	0,00%	0,00%	0,00%
Allisonella	0,00%	0,00%	0,02%
Shuttleworthia	0,00%	0,00%	0,00%
Barnesiellaceae Family	0,00%	0,00%	0,00%
Atopobiaceae Family	0,00%	0,00%	0,00%
Angelakisella	0,00%	0,00%	0,02%
Fournierella	0,00%	0,00%	0,03%
Oscillospira	0,00%	0,00%	0,02%
Veillonella	0,00%	0,00%	0,01%
Epulopiscium	0,00%	0,00%	0,00%
UCG-009	0,00%	0,00%	0,02%
Methanosphaera	0,00%	0,00%	0,00%

dietary AGEs in FRENCH FRIES with BEEF STEW

CML: 7.5; CEL 13.7; MG-H1: 27.7 mg/100g

Chapter 8

Summary and general discussion

8 Summary and general discussion

The prevalence of T2DM has reached epidemic proportions worldwide[1]. The (vascular) morbidity and mortality associated with T2DM underscores the need for identification of unknown risk factors. It is commonly accepted that lifestyle factors, including diet, contribute to the development of T2DM[2]. Throughout the last decades, industrialization has changed food preparation methods, putting emphasis on preservation, safety, and quality. It is estimated that approximately 80-90% of foods used at home are semi-processed[3], which mainly involves some sort of heat processing. As a result, modern foods represent a significant source of AGEs. Due to the pathophysiological role of AGEs in the development of T2DM and its vascular complications, the potential role of dietary AGEs herein has received increasing interest throughout the years (Figure 8.1). Although the biological effects of a diet high in AGEs has been investigated in several RCTs and observational studies, the use of outdated immunohistochemistry-based detection methods and extreme differences in cooking techniques limits the interpretation of these results, as explained in depth in Chapter 1. As such, current knowledge does not suffice to conclude whether dietary AGEs are biologically active and contribute to the development of T2DM and vascular dysfunction. The aim of this thesis was to answer this question with a complete and extensive approach using the best possible methods.

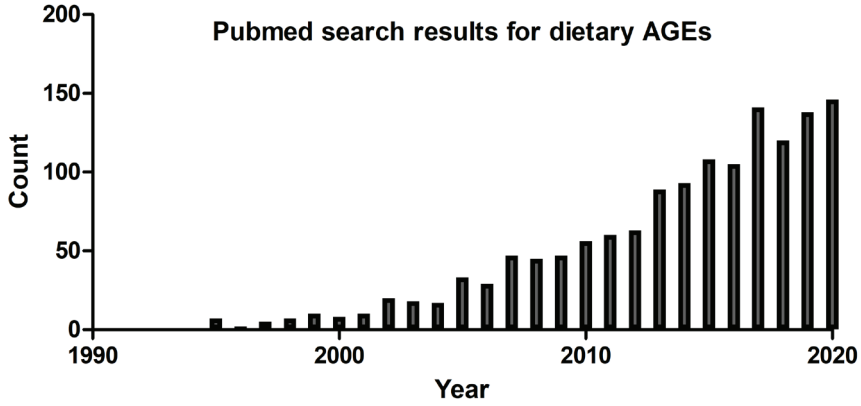


Figure 8.1 Increasing publications regarding dietary AGEs over the years.

8.1. Main findings

In Chapter 2, we investigated the effects of a 10-week baked chow diet high in AGEs on their concentrations in plasma and tissues, inflammation, and gut microbial composition in young healthy mice. Additionally, the reversibility of these effects were studied by providing an additional group of mice with the baked chow diet and a normal chow diet for two subsequent periods of 5 weeks. We observed a reversible

increase of a substantial panel of AGEs in plasma, liver, and kidney by the baked chow diet, indicating that dietary AGEs are reversibly absorbed into the circulation and organs. Additionally, inflammation increased after the baked chow diet, and gut microbial composition differed significantly between groups, effects that were reversible or discontinued by the switch to the normal chow diet lower in AGEs.

In Chapters 3, 4, and 5 we investigated cross-sectional associations between habitual intake of dietary AGEs CML, CEL, and MG-H1 and arterial stiffness, generalized microvascular function, insulin sensitivity, beta-cell function, and glucose metabolism status in the relatively large population-based cohort of The Maastricht Study. Although higher habitual intake of MG-H1 was associated with better beta-cell glucose sensitivity, we generally observed no associations between dietary AGEs and any of the other outcomes.

In Chapter 6 and 7, we investigated the effects of a specifically designed isocaloric and macronutrient-matched 4-week diet low and high in AGEs in abdominally obese individuals in a double-blind parallel-design RCT. There were marked differences in daily CML, CEL, and MG-H1 intake between groups, leading to increased free AGEs in plasma and urine after the high compared to low AGE diet. Despite this, we observed no changes in insulin sensitivity, insulin clearance, beta-cell function, macro- and microvascular function, all but one inflammatory marker, and lipid profile in both groups. Additionally, there were no changes in gut microbial diversity and richness, and overall gut microbial community structure after the low compared to high AGE diet. While we did observe both an increase and decrease in abundance of five specific low-abundancy bacterial genera, the impact might be limited, as we observed almost no other effects of the low compared to high AGE diet.

8.1.1. Dietary AGEs and their levels in plasma and urine

AGEs in plasma have been linked to many adverse health outcomes, including development of T2DM and cardiovascular mortality[4-6]. The observation that dietary AGEs from a single meal also increase AGEs in plasma[7] has opened the debate whether AGEs of dietary origin also contribute to these adverse health outcomes. While dietary AGEs increase their levels in plasma, this does not necessarily mean that they contribute to the pathophysiological mechanisms that underlie the associations between plasma AGEs and adverse health outcomes. In fact, plasma AGEs are derived from both endogenous and exogenous sources, and the association with adverse health outcomes may result from any of these sources.

Therefore, in Chapter 2, we investigated the effects of a 10-week baked chow diet high in AGEs on both free- and protein bound levels of CML, CEL, and MG-H1 in

plasma and tissues of healthy young mice. As expected, we observed an increase of all AGEs in plasma, liver, and kidney after the baked chow diet, except for free CEL in liver. Furthermore, the increase in AGEs in plasma, liver, and kidney were fully reversible by switching to a normal chow diet lower in AGEs. This suggests that any biological effects that are associated with a high AGE diet are reversible as well.

Surprisingly, in the animal study in Chapter 2, in addition to free AGEs, all protein-bound AGEs in plasma, and protein-bound CML in kidney were also increased after the baked chow diet. In the human trial in Chapter 6, only free AGEs in plasma were increased after the high compared to low AGE diet. The latter is in line with the hypothesis that dietary AGEs enter the circulation in their free form, and agrees with previous observational and experimental trials in humans[8, 9]. We hypothesize that the increase in protein-bound AGEs in the animal study results from endogenous formation rather than direct uptake of protein-bound AGEs from the gastrointestinal tract. Potentially, this is due to the baking procedure not only increasing AGEs, but also reducing micronutrient bioavailability or forming toxic substances such as dicarbonyls. In line with this, Birlouez-Aragon et al. also reported higher levels of protein-bound CML in plasma of individuals consuming a grilled/fried diet compared to those consuming a steamed diet for 4 weeks[10]. The increase in plasma protein-bound AGEs in the animal study in Chapter 2, and in humans in the study by Birlouez-Aragon et al. may contribute to the biological effects observed in these studies. However, this is not attributable solely to dietary AGEs, as we do not see in our trial (Chapter 6) an increase in protein-bound AGEs in plasma when dietary AGE intake is increased without drastically altering food preparation methods[8]. As such, we conclude that a diet high in AGEs, not solely based on largely different preparation methods, leads to an increase of free AGEs in plasma and urine.

8.1.2. Dietary AGEs and arterial stiffness

Formation of AGEs in vivo has been linked to development of arterial stiffness via mechanisms that include AGE crosslinking[11], RAGE stimulation[12], and reducing NO-bioavailability[13]. However, these mechanisms do not directly apply to AGEs of dietary origin[14], and a role of dietary AGEs in the development of arterial stiffness may be less expected. In line with this, a previous RCT showed no effect of a diet low or high in AGEs on cfPWV[15], and a cross-sectional observational trial showed no association of dietary AGEs with cfPWV, but there was an association with pulse pressure[16]. However, as these studies were performed in small groups of highly selected participants, estimated only CML intake using ELISA, and measured only aortic stiffness, more research was needed to elucidate the potential role of dietary AGEs in arterial stiffness.

These limitations were circumvented in Chapter 3, where we investigated whether the habitual intake of dietary AGEs CML, CEL, and MG-H1, with a reference period of one year, were associated with arterial stiffness measured regionally at the aorta (cfPWV) and locally at the carotid artery (carotid DC and carotid YEM) in a relatively large population-based cohort. Intake of dietary AGEs were not associated with any of the arterial stiffness measurements before and after extensive adjustment for several potential confounders. Considering the reference period of the FFQ, this may suggest that habitual intake of dietary AGEs does not contribute to AGE-crosslinking in the arterial wall. To overcome the potential shortcomings of an FFQ to accurately assess dietary AGE intake, and investigate the effects of short-term modulation of AGE intake on arterial stiffness, in Chapter 6, we investigated the effect of a specifically designed 4-week low and high AGE diet on the same measurements in an RCT. In agreement with the observational trial, we found no effect of a diet low or high in AGEs on any measurement of arterial stiffness. Although a 4 weeks intervention study may be too short to allow for structural changes in the arterial wall, these results suggest that dietary AGEs do not contribute to macrovascular endothelial dysfunction as an underlying mechanism of arterial stiffness. This is in line with results from Chapter 3 and 6 were we found no association between dietary AGEs and plasma biomarkers of endothelial dysfunction and NO-dependent measurements of endothelial function. The combined results from this observational trial and RCT strongly suggest that short-term modulation and habitual intake of dietary AGEs does not contribute to arterial stiffness in humans.

8.1.3. Dietary AGEs and generalized microvascular (endothelial) function

Similar to the role of AGEs in arterial stiffening (i.e. macrovascular dysfunction), AGEs may be important drivers of microvascular dysfunction[17, 18]. However, also similar to AGEs in arterial stiffening, the suggested mechanisms that link AGEs to microvascular dysfunction, i.e. stimulation of RAGE and quenching of NO, occur when AGEs are formed in vivo. Studies regarding the contribution of dietary AGEs to microvascular function were mainly limited to plasma biomarkers of endothelial dysfunction and showed inconsistent results. However, a recent meta-analysis suggested that, overall, a diet low in AGEs is associated with lower levels of circulating VCAM-1 levels, a reflection of better microvascular function[19]. In contrast, an RCT showed no effect of a diet low or high in AGEs on a measurement of in vivo microvascular function[20]. It is unknown whether the associations between dietary AGEs and plasma biomarkers of endothelial dysfunction are solely attributable to dietary AGEs due to the extreme differences in cooking methods employed in these studies. Furthermore, due to the large heterogeneity of the microvascular bed, a conclusion on the effects of dietary AGEs on generalized microvascular function could not be drawn.

In Chapter 4, we circumvented these limitations by investigating associations between AGE intake from the habitual diet and generalized microvascular function. To this end, we employed an extensive and unique battery of microvascular measurements consisting of plasma biomarkers of endothelial dysfunction, retinal vessel calibers, albuminuria, and in vivo endothelial function assessed by the skin microvascular response to heating and the retinal microvascular response to flicker-light. Overall, we observed no associations between the dietary AGEs CML, CEL, and MG-H1 and the microvascular outcomes, although there was a small, likely irrelevant, but statistically significant association between greater intake of CEL and lower endothelial dysfunction plasma biomarker sum score and greater flickerlight-induced retinal venular dilation. Likewise, in our RCT in Chapter 6, we did not find any effects of a 4-week diet low or high in AGEs on microvascular outcomes, additionally including insulin-induced microvascular recruitment in muscle tissue. The apparent discrepancy between our observational and experimental studies and the meta-analysis of 5 trials[19] showing an association between a diet low in AGEs and lower circulating VCAM-1 levels may be explained by several factors. Firstly, it should be noted that only 3 out of 5 included trials reported a decrease in VCAM-1 after a low compared to a high AGE diet. Of these trials, one was executed in patients on peritoneal dialysis, and these findings cannot be extrapolated to our participants[21]. Although the other two trials included individuals that were more comparable (healthy[22] or had two or more symptoms of the metabolic syndrome[23]), both had an open-label design, which may have influenced their results. Additionally, modulation of dietary AGEs in these trials was achieved through differences in cooking methods (i.e. steaming vs. grilling), and the effects of the intervention diets can therefore not be attributed to dietary AGEs alone. Our observation that we found no significant link between both habitual intake of dietary AGEs and modulation of dietary AGEs through a specifically-designed dietary intervention and plasma biomarkers of endothelial dysfunction is supported by the null findings regarding all other microvascular outcomes. As such, our results strongly suggest that short-term modulation but also habitual intake of dietary AGEs does not contribute to generalized microvascular (endothelial) function in humans.

8.1.4. Dietary AGEs and insulin sensitivity

In 2010, Birlouez-Aragon et al. were the first to report an increase in insulin resistance after a 1-month heat-treated diet high in AGEs in young, healthy students[10]. Since then, interest in restricting AGE intake to improve insulin sensitivity and possibly prevent T2DM has increased and several, mostly small, trials in healthy individuals and patients with T2DM ensued. However, results were inconsistent and diets were mostly not matched for energy and macronutrients. Furthermore, insulin sensitivity in

these studies was determined by HOMA and not the gold standard hyperinsulinemic clamp. Importantly, all studies so far employed largely different cooking methods to modulate dietary AGEs. In 2016, these limitations were partly circumvented by de Courten et al. in their crossover trial[8], who showed a net improvement of 2.1 mg/kg/min after a 2-week isocaloric and macronutrient-matched low compared to high AGE diet. However, similar to previous studies, differences in dietary AGEs were achieved by matching low and high AGE products based on differences in preparation methods. As such, before short-term restriction of dietary AGEs is suggested as a preventative treatment for T2DM, these findings should be replicated and extended upon using a parallel design RCT, and a longer dietary intervention that does not solely rely on food preparation methods to modulate dietary AGEs.

As such, we investigated the effects of a specifically designed macronutrient-matched, isocaloric 4-week low and high AGE diet on insulin sensitivity, determined by the hyperinsulinemic clamp, in abdominally obese individuals in a parallel-design RCT in Chapter 6. We found no change in insulin sensitivity after the low or high AGE diet. These observations are not in line with those of de Courten et al., despite our studies being most comparable. However, there were still some differences. Participants of the de Courten trial were on average slightly younger and more insulin sensitive, although the latter cannot be compared directly due to small differences in the hyperinsulinemic clamp protocol[24]. Potentially, these participants are more susceptible to dietary AGE-induced effects on insulin sensitivity. Interestingly, the difference in daily intake of AGEs between groups, calculated in both studies using our UPLC-MS/MS database, was greater in the deAGEing trial than in the trial by the Courten et al. (Figure 8.2). If anything, we expected a greater difference in insulin sensitivity. Most importantly, our low and high AGE diet were designed as stand-alone diets, not consisting of matched food products and not solely relying on food preparation methods to achieve differences in dietary AGEs. As such, this reduces the possibility that these differences in preparation methods has confounded our results.

In Chapter 5, we investigated the associations between habitual intake of dietary AGEs and insulin sensitivity, determined by the OGTT-derived Matsuda index, in the population-based cohort of The Maastricht Study. In line with our findings in the RCT in Chapter 6, we observed no association between habitual intake of dietary AGEs and insulin sensitivity. These combined findings strongly suggest that a 4-week diet low or high in AGEs, but also the habitual diet, do not influence insulin sensitivity in humans.

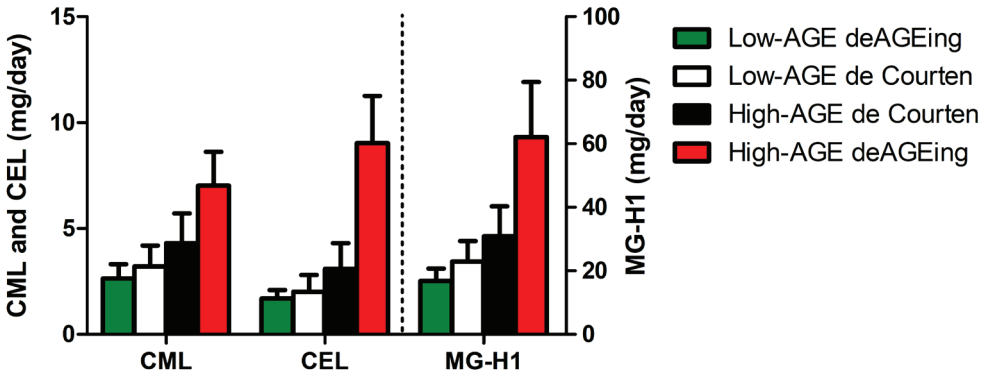


Figure 8.2 daily intake of dietary AGEs, estimated by dietary logs, in the low and high AGE groups of the deAGEing and de Courten trial. Please note that the values displayed on the right Y-axis apply to MG-H1 only.

8.1.5. Dietary AGEs and beta-cell function

Insulin-producing cells exposed to AGEs in vitro show reduced insulin secretion via mechanisms that include mitochondrial dysfunction[25-27]. This may also extend to exposure to dietary AGEs in vivo, but results between animal and human studies are inconsistent. While male Sprague Dawley rats fed a 24-week baked chow diet high in AGEs showed reduced first-phase plasma insulin levels during an intravenous glucose tolerance test (IVGTT)[27], a 2-week diet high compared to low in AGEs did not change first-phase plasma insulin levels during an IVGTT in humans[8]. However, both studies did not estimate true insulin secretion by c-peptide deconvolution, and did not adjust for insulin sensitivity. Additionally, dynamic markers of beta-cell function, such as beta-cell glucose sensitivity, were not addressed in these studies.

These limitations were addressed in Chapter 6, where we investigated the effects of a 4-week diet low or high in AGEs on both static and dynamic markers of beta-cell function during a hyperglycemic clamp. However, we found no change in insulin secretion, estimated with c-peptide deconvolution, during both the first-phase insulin secretion response as the second phase. Additional adjustment for insulin sensitivity derived from the hyperinsulinemic clamp did not materially change these findings. Additionally, beta-cell glucose sensitivity, reflecting the ability of the beta-cell to react to changes in plasma glucose[28], was not statistically different after the low compared to high AGE diet. These findings are in line with those in the aforementioned trial in humans[8]. In Chapter 5, we also investigated the association between habitual intake of dietary AGEs and both static and dynamic markers of beta-cell function during an OGTT in a population-based cohort. While we observed no association between habitual intake of dietary CML, CEL, and MG-H1 with the c-peptidogenic index, a prospectively-validated estimate of static beta-cell function[29], nor with the dynamic

beta-cell function outcomes potentiation factor and rate sensitivity, we observed a consistent association between greater habitual intake of MG-H1 and better beta-cell glucose sensitivity.

The mechanism behind this association between MH-H1 and better beta-cell glucose sensitivity and its relevance to human health remains to be determined. The accumulation of dietary AGEs in several organs of mice has been demonstrated by the use of 13^{C}-labeled CML[30], but whether this accumulation also applies to the pancreas has not been investigated. Additionally, factors that could influence beta-cell function, such as inflammation, endothelial function, or lipid profile, all remained largely unchanged in both our RCT in Chapter 6 as well as in the well-controlled RCT by de Courten et al[31]. Furthermore, the association between greater intake of MG-H1 and better glucose sensitivity did not translate into decreased prevalence of prediabetes or T2DM, nor was there an association between greater intake of MG-H1 and better glucose measures. While this also could result from the cross-sectional design of the current data, it becomes clear that large prospective studies are needed to imply a causal relation between habitual intake of AGEs and beta-cell function.

Combined, our findings strongly suggest that short-term modulation of dietary AGEs does not influence beta-cell function in humans. Habitual intake of AGEs shows no association with most measures of beta-cell function, but a beneficial association between intake of MG-H1 and beta-cell glucose sensitivity cannot be excluded.

8.1.6. Dietary AGEs, the liver, inflammation, and lipid profile

The clinical manifestations of NAFLD and NASH, including insulin resistance, atherogenic dyslipidemia, and low-grade inflammation, have also been linked to a high AGE diet[19]. Furthermore, hepatic accumulation of AGEs seem involved in the severity of NAFLD[32, 33]. As such, dietary AGEs may exert biological effects through an interaction with the liver, possibly via NAFLD progression.

In line with this thought, we observed a reversible increase in the free AGEs CML, CEL, and MG-H1 in the liver of mice after a baked chow diet high in AGEs in Chapter 2. Additionally, there was an increase in an inflammatory sum score in these mice, which was largely attributable to a decrease in circulating IL-10. However, whether this resulted from hepatic inflammation is unknown. Only free AGEs, and not protein bound AGEs, were increased in the liver of mice fed the baked chow diet. Furthermore, as we did not perform histochemical analyses, it is unknown whether any liver damage occurred. In contrast, we did observe increased levels of protein bound CML in kidney after the baked chow diet, indicating that this is a potential

source for the increase in inflammation, possibly via RAGE dependent pathways.

However, in our RCT in humans with high risk of fatty liver (Chapter 6), we observed no deterioration in insulin resistance, inflammation, or lipid profile after the high compared to low AGE diet. If anything, there was an improvement in overall inflammation after the high AGE diet, as apparent from increased adiponectin and decreased monocyte levels. Additionally, the effect of the low or high AGE diet on the liver were limited, as gamma-GT and circulating e-Selectin, both of which are independently associated with intra-hepatic lipid content[34] and NAFLD severity[35], remained unchanged during both interventions. However, this is only indirect evidence since we did not measure intrahepatic lipid content or performed histological examination.

The lack of increased inflammation after the high compared to low AGE diet in Chapter 6 is striking, especially when compared to our own findings in mice in Chapter 2. Additionally, several trials in humans have reported increased inflammatory markers in plasma after a high compared to low AGE diet[19]. However, this may be attributed to the largely different cooking methods used to modulate dietary AGEs in these studies. The limitations of using a baked chow diet as a model for high dietary AGEs in mice also apply to employing largely different cooking methods in human trials. Although it seems that a grilled versus cooked diet may lead to biological effects, it is then unknown to what extent these are attributable to dietary AGEs, or due to other changes that have occurred with these preparation methods. Our findings in humans suggest that a short term diet high in AGEs does not induce inflammation. Whether these findings also apply to habitual intake of dietary AGEs, or patient groups, deserves further investigation.

8.1.7. Dietary AGEs and gut microbiota composition

The gut microbiome is increasingly recognized as an important determinant of health[36-38]. As dietary AGEs mostly reach the colon undigested, they can potentially act as substrates for gut microbes and alter microbiota composition. In line with this, several murine experimental studies report a change in gut microbiota composition after a baked chow diet high in AGEs[39-42]. However, results regarding individual species are inconsistent, possibly due to differences in host-species compositions, chow baking protocols, and microbiota analytical methods (qPCR versus 16s rRNA). Furthermore, in humans only two trials have been performed, which both show limited external validity due to highly selected participant groups, and modulated dietary AGEs by largely different food preparation methods.

To investigate a causal link between gut microbial composition and dietary AGEs, we

investigated in Chapter 2 the reversible effects of a baked chow diet high in AGEs on gut microbial composition in healthy young mice. From 5 weeks and onward, mice fed the baked chow diet high in AGEs showed a difference in general gut microbial community structure compared to mice fed the standard chow diet. Specifically, of several species showing different abundance after the baked chow diet, an enrichment in *Dubosiella* spp. dominated these alterations. In a different group of mice, the changes induced by the baked chow diet after 5 weeks were almost completely attenuated after 5 subsequent weeks of the standard chow diet. These results strongly suggest that a baked chow diet high in AGEs leads to a reversible change in gut microbiota content of mice. Although this provides a proof-of-concept, the relevance of these changes to humans, especially the enrichment in *Dubosiella* spp., cannot be answered with this animal study.

To this end, we also investigated the effects of a 4-week diet low or high in AGEs on gut microbiota of humans in Chapter 7. We observed no difference in gut microbial richness and diversity after the low compared to high AGE diet. Additionally, there were no differences in overall microbial community structure after the low compared to high AGE diet. Although we did find an enrichment in the genera *Tyzzerella* and *Family_XII_UCG-001*, and a contraction in the genera *Negativibaccillus, Oscillibacter*, and *Anaerostipes* after the low compared to high AGE diet, all these comparisons lost statistical significance after adjusting for multiple testing. Furthermore, the implications of these potential short-term changes in gut microbiota composition to human health are limited, as we observed very limited biological effects of the low or high AGE diet in general.

8.2. Methodological considerations

8.2.1. Assessment of dietary AGE intake

Diet is commonly accepted to play a major role in the etiology of several chronic diseases. However, although there are several methods to determine dietary intake, there is no gold standard. As such, the potential methodological limitations of the employed methods should be considered. In this thesis, habitual food intake was assessed by means of a validated 253-item semi-quantative FFQ that was specifically designed for use in the Maastricht Study[43]. FFQs are prone to recall bias, where participants may incorrectly remember their food intake and portion sizes, potentially leading to under- or overestimation of nutrient intake[44]. This becomes clear when comparing intake of individual food groups by the Maastricht FFQ and repeated 24-hour recalls. While larger food groups, such as grains, bread, and meats are within 10% difference between methods, this is not necessarily the case for smaller but relevant food groups. For example, intake of high AGE nuts and snacks estimated by

the FFQ was almost double the intake based on repeated 24-hour recalls[43]. This has important implications for the assessment of dietary AGE intake, as this is calculated from the sum of the individual food items within their respective groups. To date, no FFQ has been validated for the assessment of dietary AGEs. However, since its publication, both the Maastricht FFQ and our UPLC-MS/MS dietary AGE database have been adapted to complement each other. On the one hand, the FFQ has been updated with several high AGE food products that are commonly-consumed in the burgundy population of Limburg. On the other hand, the dietary AGE database has been updated with additional food products that were already present in the FFQ but not in the database (Table 8.1).

Table 8.1 AGE-content of commonly-consumed high AGE products in Limburg, as compared to other products lower in AGEs [45]

Food	Specification	mg CML /100g	mg CEL /100g	mg MG-H1 /100g
Black pudding	With crushed bacon, fried 16 minutes	4.82	7.71	63.01
Beef stew	Hachee (Struijk)	5.78	14.48	57.16
Chips	Deep fried 5 minutes	0.10	0.08	0.81
Potato	Boiled 7 minutes	0.01	0.00	0.24

Another consideration of using the Maastricht FFQ to investigate associations between dietary AGEs and outcomes is the reference period for dietary intake, which is one year. The relevant window of exposure for dietary AGEs to elicit biological effects, if any, is unknown. This is further complicated when exposure to dietary AGEs on the very long term cannot be accurately assessed by the FFQ if an individual's dietary pattern changed throughout the years. Alternatively, associations between dietary AGEs and outcomes are potentially flawed when the habitual diet is deviated from in the days/weeks prior to the outcome measurement. However, it should be noted that our results in Chapter 2, 3, and 4 were generally unchanged when we performed sensitivity analyses either excluding participants with apparent CVD or participants with previously diagnosed T2DM, whom are likely to have received dietary advice from a health care professional.

Another potential limitation lies within the nature of the Maillard reaction itself: heat is the most potent driver of AGE formation in food. Although all products that are included within our dietary AGE database were prepared according to the manufacturer's instructions, preparation conditions by an individual at home may vary, and thus the ultimate quantity of AGEs in food may deviate from that what is present in the database. This is one of the strengths of the deAGEing trial, in which a low and high AGE diet was specifically-designed and a large difference (3-5-fold) in AGE intake was confirmed by biomarkers.

8.2.2. Attributing effects solely to dietary AGEs

It is difficult to assess relationships between single (non) nutrients and health outcomes. Nutrients are consumed as a combination of foods, which in turn reflects genetic, cultural, social, health, environmental, lifestyle, and economic determinants[46]. Especially in the observational cohort study in Chapter 2, 3, and 4, residual confounding cannot be excluded, although we applied extensive adjustment for several possible confounders, including the overall diet. Notably, increasing intake of particular foods generally leads to compensatory changes in other dietary components. This became apparent in chapter 6, and 7 where our intervention diets, designed to be isocaloric- and macronutrient-matched, showed disparities in some micronutrients.

Despite not being able to exclude potential confounding effects of subtle differences in micronutrients, dicarbonyls, or even other Maillard reaction products, the dietary interventions used in our RCT represent a practical example of a low and high AGE without resorting to extreme differences in cooking techniques. Although one can argue that biological effects of dietary AGEs should be investigated by supplementing the diet with AGEs, for example in powdered form, the implications of this approach are limited as a subsequent translation into daily practice is not possible.

8.2.3. External validity

Caution should be exercised when translating our results to other populations. Although chapter 2, 3, and 4 represent findings from a population-based cohort, these results do not necessarily apply to other cohorts. Primarily, participants of the Maastricht study are, by design, aged 40-75. In addition, the consumption of dietary AGEs in this traditionally burgundy population, and especially the contribution of different food groups herein, may differ from those in other countries[47]. Finally, although we found no consistent interactions by glucose metabolism status in our observational studies, participants of the intervention trial were healthy and potential effects of a diet low or high in AGEs in patients with T2DM cannot be deduced from the current results. In line with this, we cannot exclude that in patients with impaired kidney function dietary AGEs may exert biological effects due to decreased clearance from the circulation. In Chapter 4, greater habitual intake of CEL was associated with lower insulin sensitivity in participants with impaired kidney function (n = 111, median eGFR 55 ml/min/1.73m^2).

8.2.4. A critical appraisal of baked chow as a model of a high AGE diet

The limited consequences of a (short term) diet high in AGEs on glucose metabolism, vascular function, and gut microbiota composition in humans is in stark contrast to findings in mice. For example, we report increased inflammation and a change in gut microbiota composition in mice fed a baked chow diet high in AGEs[48], and others have reported decreased beta-cell function[27] using a similar approach. Naturally, findings in mice cannot be directly extrapolated to humans. However, the validity of the "baked chow diet" as a model of high AGE intake should be reviewed, as it is relatively unknown whether the intake of AGEs by mice supplied a high AGE diet is comparable to that what humans would consume. Using a "human equivalent dose calculation", based on body surface area, AGE dosages provided to mice can be converted to the human situation. The daily intake of CML in the deAGEing trial and The Maastricht Study is known, and when converted to intake per kg of bodyweight, the low AGE group and quartile 1 of The Maastricht Study consumed 0.03 mg CML/kg_{bw}/day (Figure 8.3). Those in the high AGE group and quartile 4 of The Maastricht study consumed 0.08 mg CML/kg_{bw}/day. However, when these calculations are performed in mice the intake of CML, even in the standard chow group, is far greater than in the highest human consumers. Using a conversion factor to translate a dosage in mice to humans (3/37), the intake of CML in our standard and baked chow group was 0.18 mg CML/kg_{bw}/day and 0.48 mg CML/kg_{bw}/day, respectively. This comparison demonstrates that CML levels in baked chow are too high, even though we only baked our chow for 2 hours at 160 °C. Interestingly, even the CML intake via unbaked chow were threefold higher than consumed by individuals of the high AGE diet in the deAGEing trial. By comparison, Grossin et al. provided a CML-enriched diet to mice, achieving dosages not feasible by baking only. Only mice consuming 1.43 and 3.03 mg CML/kg_{bw}/day, and not mice consuming 0.70 mg CML/kg_{bw}/day, showed RAGE-dependent arterial stiffening after 9 months[49]. As such, these comparisons suggest that the AGE-levels of baked chow diets are too high in comparison to what humans could realistically consuming during a high AGE diet, and that only levels beyond the physiological range lead to detrimental biological effects. This adds another difficulty to translating findings in mice to humans.

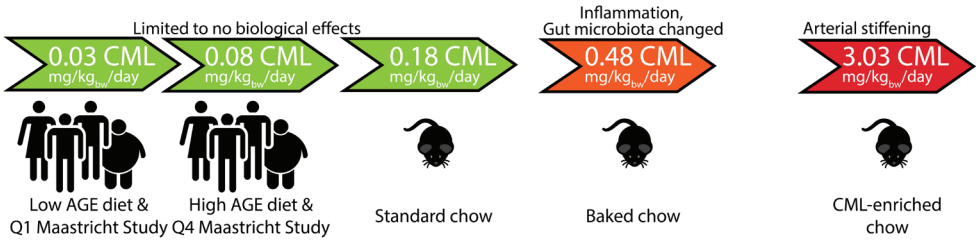


Figure 8.3 Daily intake of CML (mg/kg_{bw}/day) in participants of the deAGEing trial, quartiles of CML intake of The Maastricht Study, and mice fed the standard and baked chow diet. Furthermore, the intake of CML during a CML-enriched chow diet is shown[49]. Intake of CML in mice was transformed into a human equivalent dose based on a (3/37) conversion factor[50].

8.3. Future directions for dietary AGE research

In this thesis, we did not observe associations between habitual intake of dietary AGEs and arterial stiffness, generalized microvascular dysfunction, insulin sensitivity, and most measures of insulin secretion in humans, nor were there effects of a 4-week diet low or high in AGEs on these outcomes. Although these results strongly rule against dietary AGEs posing detrimental effects to health, these findings should be confirmed in large longer-term prospective studies before drawing a definite conclusion. Our findings in The Maastricht Study need to be interpreted with its main limitations in mind, being its (current) cross-sectional design, the assessment of dietary AGEs via FFQs, and the limited size of our dietary AGE database. Similarly, our 4-week dietary intervention may simply prove too short to induce statistically significant changes in clinical parameters. Additionally, even if we had observed effects of short-term modulation of dietary AGE intake, there is no guarantee that a continued intervention will lead to a change in hard clinical outcomes on the long term. To increase cost-effectiveness, a logical next step would be to investigate biological effects of dietary AGEs using large longitudinal observational trials where information on dietary intake is already collected. Intake of dietary AGEs can be determined relatively easy in these cohorts by using our UPLC-MS/MS dietary AGE database[45]. An example is the large European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. It would be interesting to investigate whether greater intake of dietary AGEs results in increased risk of developing prediabetes and T2DM in a large prospective cohort such as EPIC. In this cohort higher habitual intake of dietary AGEs has already been associated with greater risk of gallbladder cancer but reduced risk of hepatocellular carcinoma[51] and also with greater weight gain[47]. These associations both provide directions for future research. The association with weight gain is interesting, as this reflects an alternative pathway for dietary AGEs to be involved in the T2DM epidemic. On the one hand, foods high in dietary AGEs may be more energy dense, leader to higher energy intake[10]. On the other hand, in vitro work suggest that certain cell lines exposed to free CML release serotonin[52, 53], an important regulator of satiety[54]. The association with risk of some cancer types is particularly interesting regarding the increase in urine levels of CEdG after the low compared to high AGE diet in Chapter 6. The formation of N^2-carboxyethyl-2′-deoxyguanosine (CEdG) by methylglyoxal, a highly-reactive precursor to AGEs, is considered highly mutagenic[55, 56]. However, the underlying mechanism behind the increase of urinary CEdG, and its relevance, is unknown and requires more research.

The construction of our UPLC-MS/MS-based dietary AGE database has opened the possibility to reliably quantify exposure to dietary AGEs[45]. Furthermore, compared to immunohistochemistry-based databases[57], AGE intake determined by UPLC-MS/

MS is expressed as mg/day instead of arbitrary kilounits/day and can be compared between study populations. Nonetheless, the methods used to assess dietary AGE intake in future studies need improvement. In large cohort studies, food intake is generally determined by FFQs. However, to date, no FFQ has been validated for determination of AGE intake. The Maastricht FFQ, but certainly also other FFQs, may under- or overestimate intake of individual food groups and lead to inaccurate estimates of dietary AGE intake. Ideally, the Maastricht FFQ should be validated specifically for dietary AGE intake, but a gold standard method to determine food intake is not available. As an alternative, estimates of habitual dietary AGE intake by different methods (i.e. FFQ, food diaries, 24-hour recalls) over the same reference period should be compared to gain insight in discrepancies between methods. Additionally, when investigating exposure to dietary AGEs on the short term, levels of free AGEs in plasma or urine can be included as biomarkers.

8.4. Conclusion

The work presented in this thesis demonstrates that a diet high in advanced glycation endproducts is unlikely to contribute to the development of type 2 diabetes mellitus or vascular dysfunction.

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dietary AGEs in KNÄCKEBRÖD with CANNED SALMON

CML: 2.1; CEL 4.2; MG-H1: 24.4 mg/100g

Chapter 9

Valorization
Nederlandstalige samenvatting
Dankwoord
Scientific output
Curriculum vitae

9 Impact (formerly known as valorization addendum)

9.1. Luckily, dietary AGEs do not worsen risk factors for T2DM.

Food processing techniques and food composition have changed massively throughout the 20th century. It is estimated that approximately 80-90% of all foods used for cooking are now already semi-processed[1]. This often involves some form of heat treatment. Many beneficial effects are associated with heat treatment, such as inactivating food borne pathogens, prolonging shelf life, increasing taste, smell, and texture, improving digestibility, but also increased consumer convenience as many of these foods are ready-to-eat[2]. However, resulting from the Maillard reaction that occurs within protein- and sugar rich heat-treated foods, AGEs are formed. As such, the modern diet represents a significant contribution to our exposure to AGEs. This may even start at a very early age, as heat-treated infant formula may contain up to 670-fold more AGEs than human breastmilk[3]. Other commonly-consumed high AGE products include bread, heat-treated cereals, meat, and confectionaries.

AGEs are most-known from their formation within the body, a process that occurs naturally with ageing[5, 6] but is accelerated in T2DM due to the presence of hyperglycemia[7]. In this context, AGEs are involved in the development of both the cardiovascular complications of T2DM[8-14] but also of T2DM itself[11, 15, 16]. From the observation that dietary AGEs may be absorbed into the circulation[17, 18], and that their presence in our modern diet is significant, it has been hypothesized that dietary AGEs may also contribute to the many biological consequences that are described for in-vivo formed AGEs. Particularly, a diet high in AGEs may be a risk factor for the development of insulin resistance and ultimately T2DM[19-23]. Interestingly, based on these previous studies, some authors have suggested that restricting dietary AGE intake may present a novel preventative treatment for the development of T2DM. Moreover, one author wrote a book on the “toxicity” of dietary AGEs and a guide on how to reduce their intake.

However, as explained in detail in Chapter 1 and 8, this conclusion may be too strong, and the work provided in this thesis, with its limitations and external validity in mind, does not support the hypothesis that dietary AGEs contribute to the development of T2DM. While this may be regarded as disappointing, as we were not able to identify a new risk factor for the T2DM epidemic, results of our studies do not underscore the need to reduce AGE intake. If this would apply, large efforts at the individual level but especially at the level of food manufacturers were needed. As explained above, AGEs are widely present in our modern diet, especially in products that are highly palatable, and individuals at risk for diabetes would have to consume less-desirable

food products that contain less AGEs. On the other hand, food manufacturers would have to construct preparation methods that retain the many beneficial effects of the Maillard reaction while simultaneously limiting AGE formation. Doing so would require extensive knowledge on the factors that drive AGE-formation in foods.

9.2. Free AGEs in urine and plasma are a short-term reflection of dietary AGE intake

From our repeated analyses of free pyrraline in urine during a 3-day low and high AGE diet in the Addendum to Chapter 6, it becomes evident that free AGEs in urine are rapidly influenced by AGE intake. Moreover, after a 4-week diet low compared to high in AGEs in Chapter 6, the increase in free AGEs in plasma is sustained after an overnight 12-hour fast. This suggests that free AGEs in plasma and urine may serve as a sensitive marker of dietary AGE intake on the very short term. However, this also has consequences for studies in which free AGEs in plasma or urine serve as an outcome of interest. Participants in clinical trials often visit the lab in the fasted state, and remain as such during the remainder of the day. Thus, it is likely that participants treat themselves to a nice (and therefore high AGE) meal the evening prior to their lab visit. Although the randomization process in a randomized controlled trial should distribute this occurrence equally over treatment arms, this will lead to inaccuracies of treatment effects. This in turn increases the number of individuals needed to detect a statistically significant difference between groups. Trials investigating the effects of an intervention, be it dietary, pharmaceutical, or exercise, should standardize intake of dietary AGEs before collecting blood or urine samples at baseline or follow-up. Although several trials investigating the effect of a low or high AGE diet on insulin sensitivity applied some sort of dietary restriction days/weeks before the baseline visit, such as restricting junk food, we are to our knowledge the only to prescribe a "normal" AGE diet two days prior to the baseline visit.

9.3. Not publishing null findings is a threat to scientific progress

The work provided in this thesis demonstrates very limited biological effects of dietary AGEs in humans, which is in stark contrast compared to several previous studies. Although this can partly be explained by methodological differences between studies, another potential explanation is that previous null findings have not been published. In a 2007 analysis of a sample of published studies over 22 disciplines, 85.9% of results were positive and in line with the hypothesis[24]. Thus, it goes without question that a serious proportion of null findings are not published. This problem is termed positive-outcome bias, and is a threat to scientific progress. The overabundance of positive findings inflates effect size estimates in meta-analyses[25] and consequently wastes recourses as researchers try to replicate non-existent findings or conduct

nonsensible follow-up studies. Although positive-outcome bias may occur at the level of a journal or of authors, it is suggested that once a manuscript is submitted, acceptance rate is similar for positive and null results[26]. As such, authors are suggested to be mainly responsible.

Positive-outcome bias occurs when authors selectively report their outcomes, or when the hypothesis is changed after the results are already known. When not all outcomes are in line with the original hypothesis, providing a theoretical background for this discrepancy is often difficult. As such, it is easier to draft a manuscript when null findings are left out. Additionally, articles with positive results are approximately two times more likely to be cited[27], which is termed citation bias, and provides another potential rationale for authors to leave out null findings. In this thesis, we report almost exclusively null findings. Our results are not in line with several other previous studies that show biological effects of a low or high AGE diet supplied for similar durations. Our extensive panel of outcomes and combined experimental and epidemiological approach emphasizes that positive-outcome bias does not apply to the work described in this thesis. Although our findings should be validated by other groups, it is evident that our approach reduces the need for several new trials.

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De invloed van versuikerde eiwitten uit voeding op de suikerhuishouding, werking van de bloedvaten, en darmflora

Type 2 diabetes en versuikerde eiwitten

Er is sprake van een type 2 diabetes (T2D) epidemie. Sinds 1985 is wereldwijd het aantal gevallen verviervoudigd en in Nederland zal het in 2035 naar schatting bijna 1 op de 10 volwassenen treffen. T2D geeft een sterk verhoogd risico op hart- en vaatziekten en hierdoor een enorme belasting voor het individu en de samenleving. De huidige aanpak van deze epidemie schiet te kort, ook omdat niet volledig verklaard kan worden waarom er zo'n sterke toename is. Het identificeren van tot nog toe onbekende risicofactoren is nodig om nieuwe aanknopingspunten te geven voor behandelstrategieën.

Het eten van versuikerde eiwitten is mogelijk zo'n risicofactor. Versuikerde eiwitten worden in suiker- en eiwitrijke voeding gevormd wanneer deze wordt blootgesteld aan droge hitte, bijvoorbeeld bij het bakken, braden, en roosteren. Het verhitten van voeding maakt het langer houdbaar, smakelijker, en visueel aantrekkelijker, en dit wordt veelvoudig toegepast. Verhitte vlees, noot- en graanproducten bevatten de meeste versuikerde eiwitten, denk aan saté, gebrande pinda's, en ontbijtgranen.

Naast de vorming in voeding vormen versuikerde eiwitten zich ook in het lichaam door de spontane reactie van suiker met eiwitten. Dit proces treedt versneld op bij mensen met T2D door de verhoogde bloedsuikerspiegels. De ophoping van deze versuikerde eiwitten is schadelijk voor bloedvaten en draagt bij aan het verhoogde risico op hart- en vaatziekten bij mensen met T2D. De ophoping van versuikerde eiwitten lijkt daarnaast ook het risico op T2D zelf te verhogen doordat het twee belangrijke risicofactoren verslechtert, namelijk de afgifte en werking van het hormoon insuline.

De versuikerde eiwitten die wij eten zijn hetzelfde als de versuikerde eiwitten die zich vormen in het lichaam en waarvan we weten dat ze schadelijk zijn. Er zijn aanwijzingen dat versuikerde eiwitten uit voeding worden opgenomen in het lichaam, maar of ze ook bijdragen aan een verhoogd risico op T2D en problemen van de bloedvaten is onvoldoende onderzocht. Daarnaast is het de vraag via welke mechanismen dit dan gebeurt. Deze vragen staan in dit proefschrift centraal. Als het eten van versuikerde eiwitten bijdraagt aan het risico op T2D en problemen van de bloedvaten, kan dit mogelijk voorkomen worden door de inname van versuikerde eiwitten te verminderen.

Opzet

De bovenstaande vragen worden beantwoord door gegevens uit drie verschillende studies. In de muizenstudie van **hoofdstuk 2** is onderzocht of versuikerde eiwitten uit voeding worden opgenomen in het bloed en organen, of het eten van versuikerde eiwitten de darmflorasamenstelling verandert, en of het eten van versuikerde eiwitten ontstekingswaarden in het bloed verhoogt. De muizen kregen gedurende 10 weken oftewel normale muizenvoeding oftewel gebakken muizenvoeding waar veel versuikerde eiwitten in zitten. Ook was er een groep muizen die achtereenvolgens 5 weken gebakken en 5 weken ongebakken muizenvoer kreeg, om een oorzakelijk verband tussen het eten van versuikerde eiwitten en de veranderingen in uitkomsten nog aannemelijker te maken.

In een observationele cohortstudie in mensen, genaamd De Maastricht Studie, is in **hoofdstuk 3, 4,** en **5** in meer dan 3000 mensen onderzocht of diegene die van zichzelf over het afgelopen jaar dagelijks meer versuikerde eiwitten aten de bloedvaten slechter werken of dat er sprake is van een verhoogd risico op het krijgen van T2D. De dagelijkse inname van versuikerde eiwitten in deze studies is geschat middels voedingsvragenlijsten. Deze vragenlijst is gekoppeld aan onze uitgebreide voedingsdatabase, waarin van meer dan 200 producten de hoeveelheid versuikerde eiwitten staan vermeld. Specifiek gaat het om de versuikerde eiwitten N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), en N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1).

Tenslotte is in een experimentele studie in mensen, genaamd de deAGEing studie, in **hoofdstuk 6** en **7** onderzocht of versuikerde eiwitten uit voeding worden opgenomen in het bloed, de darmflorasamenstelling veranderen, ontstekingswaarden in het bloed verhogen, de werking van bloedvaten verminderen, en het risico op T2D verhogen. Twee groepen van 41 deelnemers kregen hiervoor een speciaal ontwikkeld eetschema met daarin weinig of veel versuikerde eiwitten, maar wel een gelijk aantal calorieën, dat 4 weken duurde. Deze studie levert de hoogst mogelijke bewijslast en is de beste benadering van een oorzakelijk verband.

Belangrijkste bevindingen

Versuikerde eiwitten uit voeding worden opgenomen in het lichaam

In **hoofdstuk 2** zagen we dat in muizen die gebakken muizenvoer aten de hoeveelheid versuikerde eiwitten in hun bloed en organen toenamen. Door het gebakken voer weer te vervangen voor ongebakken voer namen de versuikerde eiwitten in bloed en organen vervolgens weer af. Ook in **hoofdstuk 6** zagen we dat in mensen die een eetschema volgden met veel versuikerde eiwitten de hoeveelheid hiervan in bloed en urine toenam. Dit samen zijn sterke indicaties dat de versuikerde eiwitten die wij eten worden opgenomen in ons lichaam.

Versuikerde eiwitten uit voeding leiden niet tot problemen van de bloedvaten

De versuikerde eiwitten die vanuit voeding in de bloedsomloop worden opgenomen staan in direct contact met de binnenbekleding van bloedvaten. Deze binnenbekleding bepaalt voor een deel hoe goed de bloedvaten werken. Ondanks dit zagen we in **hoofdstuk 3** en **4** overwegend geen verbanden tussen de dagelijkse inname van versuikerde eiwitten en de werking van de allerkleinste en grote bloedvaten. Ook in **hoofdstuk 6** zagen we na een verlaging en verhoging van de inname van versuikerde eiwitten geen verandering in de werking van de allerkleinste en grote bloedvaten. Dit samen zijn sterke indicaties dat het eten van versuikerde eiwitten niet bijdraagt aan een verminderde werking van de bloedvaten.

Versuikerde eiwitten uit voeding verhogen niet het risico op T2D

In **hoofdstuk 5** zagen we dat mensen die van zichzelf dagelijks meer versuikerde eiwitten aten niet vaker T2D hadden dan mensen die minder versuikerde eiwitten aten. Dit betreft echter een momentopname, en sluit niet uit dat op langere termijn het eten van versuikerde eiwitten het risico op T2D wel verhoogt. Voor de achterliggende risicofactoren voor T2D werd echter eenzelfde beeld gezien. In hoofdstuk 5 zagen we niet dat mensen met een hogere dagelijkse inname van versuikerde eiwitten een slechtere werking of afgifte van insuline hadden. Sterker nog, mensen met hogere dagelijkse inname van MG-H1 hadden zelfs hogere afgifte van insuline dan diegene die dagelijks minder MG-H1 aten. Mogelijk berust deze bevinding echter op toeval, aangezien we in **hoofdstuk 6** geen verandering zagen in de werking of afgifte van insuline nadat de deelnemers minder of meer versuikerde eiwitten hadden gegeten. Dit alles tezamen suggereert dat het eten van meer versuikerde eiwitten niet gepaard gaat met een verhoogd risico op T2D.

Versuikerde eiwitten uit voeding veranderen wel de darmflorasamenstelling en verhogen wel ontstekingswaarden in muizen, maar niet in mensen

Twee veelbelovende mechanismen waarop het eten van versuikerde eiwitten tot negatieve gezondheidsgevolgen kan leiden is door een interactie met de darmflora en het veroorzaken van laaggradige inflammatie. Gedurende de afbraak en opname van versuikerde eiwitten vanuit de darmen staan deze namelijk in contact met de darmflora en kan er een wisselwerking optreden: enerzijds gebruiken darmbacteriën versuikerde eiwitten als energiebron waardoor ze in getalen kunnen toenemen, anderzijds ontstaan er bij dit proces afbraakproducten die op zichzelf biologisch actief kunnen zijn. In **hoofdstuk 2** was er reeds na 5 weken een verandering te zien in darmflorasamenstelling van de muizen die het gebakken voer aten. Deze verschillen in darmflorasamenstelling verdwenen nagenoeg volledig wanneer het gebakken muizenvoer weer werd vervangen door het ongebakken muizenvoer. Ook zagen we een toename van laaggradige inflammatie in de muizen die het gebakken voer hadden gegeten. Opvallend genoeg was dit in de menselijke studiedeelnemers van **hoofdstuk 6** en **7** niet het geval. Er was geen verandering in de darmflorasamenstelling of mate van laaggradige inflammatie nadat de deelnemers minder of meer versuikerde eiwitten hadden gegeten. Een mogelijke verklaring hiervoor is dat de hoeveelheid versuikerde eiwitten in gebakken muizenvoer naar verhouding te hoog is vergeleken met de hoeveelheid die mensen realistisch gezien kunnen eten. De hoeveelheid versuikerde eiwitten in het gebakken muizenvoer was namelijk 6 keer zo hoog als dat in het “hoge” eetschema van de deAGEing studie.

Conclusies

De resultaten van de studies in dit proefschrift benadrukken dat alhoewel versuikerde eiwitten uit voeding worden opgenomen in het lichaam, ze niet lijken bij te dragen aan het risico op T2D en problemen van de bloedvaten. Ook zagen we dat het eten van versuikerde eiwitten in mensen niet gepaard gaat met een toename van ontstekingswaarden in het bloed of een verandering in de darmflorasamenstelling. Nadelige gevolgen van versuikerde eiwitten uit voeding op mensen lijken daarom, tegen de verwachting in, afwezig. Opvallend is dat in muizen versuikerde eiwitten uit voeding wel een invloed hebben op ontstekingswaarden en de darmflorasamenstelling, mogelijk doordat de hoeveelheid versuikerde eiwitten in gebakken muizenvoer veel hoger is. Om die reden is het interessant om te onderzoeken of mensen met verminderde nierfunctie, die versuikerde eiwitten minder snel kunnen uitplassen, wel nadelige gezondheidsgevolgen ondervinden van versuikerde eiwitten uit voeding.

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Scientific output

List of publications

Linkens AMA, van Best N, Niessen PM, Wijckmans NEG, de Goei EEC, Scheijen JLJM, van Dongen MCJM, van Gool CCJAW, de Vos WM, Houben AJHM, Stehouwer CDA, Eussen SJMP, Penders J, Schalkwijk CG. A 4-Week Diet Low or High in Advanced Glycation Endproducts Has Limited Impact on Gut Microbial Composition in Abdominally Obese Individuals: The deAGEing Trial. International journal of molecular sciences. 2022;23(10):5328.

Linkens AM, Houben AJ, Niessen PM, Wijckmans NE, de Goei EE, Van den Eynde MD, Scheijen JL, van den Waarenburg MP, Mari A, Berendschot TT, Streese L, Hanssen H, van Dongen MC, van Gool CC, Stehouwer CD, Eussen SJ, Schalkwijk CG. A 4-week high-AGE diet does not impair glucose metabolism and vascular function in obese individuals. JCI Insight. 2022;7(6).

Linkens AMA, Houben A, Kroon AA, Schram MT, Berendschot T, Webers CAB, van Greevenbroek M, Henry RMA, de Galan B, Stehouwer CDA, Eussen S, Schalkwijk CG. Habitual intake of dietary advanced glycation end products is not associated with generalized microvascular function-the Maastricht Study. The American journal of clinical nutrition. 2022;115(2):444-55.

van Dongen KCW, **Linkens AMA**, Wetzels SMW, Wouters K, Vanmierlo T, van de Waarenburg MPH, Scheijen JLJM, de Vos WM, Belzer C, Schalkwijk CG. Dietary advanced glycation endproducts (AGEs) increase their concentration in plasma and tissues, result in inflammation and modulate gut microbial composition in mice; evidence for reversibility. Food Research International. 2021;147:110547.

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Churchward-Venne TA, Snijders T, **Linkens AM**, Hamer HM, van Kranenburg J, van Loon LJ. Ingestion of Casein in a Milk Matrix Modulates Dietary Protein Digestion and Absorption Kinetics but Does Not Modulate Postprandial Muscle Protein Synthesis in Older Men. The Journal of nutrition. 2015;145(7):1438-45.

Oral presentations

Young AGE researchers meeting (YoungAGErs) 2021 (virtual): The effect of a 4-week diet low and high in AGEs on glucose metabolism, vascular function, and markers of low-grade inflammation of abdominally obese individuals: the deAGEing trial.

Annual meeting of the European Association for the Study of Diabetes (EASD) 2021 (virtual): Habitual intake of dietary advanced glycation endproducts is not associated with generalized microvascular function: The Maastricht Study.

International Maillard Reaction Society conference (iMARS) 2021 (virtual): The effect of a 4-week diet low and high in AGEs on insulin sensitivity and secretion, vascular function, and markers of low-grade inflammation and endothelial dysfunction of abdominally obese individuals: preliminary results from the deAGEing trial.

Annual Dutch Diabetes Research Meeting (ADDRM) 2019 (Wageningen, The Netherlands): Intake of advanced glycation endproducts is not associated with arterial stiffness: The Maastricht study

Young AGE researchers meeting (YoungAGErs) 2019 (Maastricht, the Netherlands): Restriction of dietary AGEs to prevent diabetes in overweight individuals: a randomized controlled trial.

Poster presentations

International Maillard Reaction Society conference (IMARS) 2021 (virtual): Dietary advanced glycation endproducts (AGEs) increase their concentrations in plasma and tissues, result in inflammation and modulate gut microbial composition in mice; evidence for reversibility.

37th International Symposium on Diabetes and Nutrition (DNSG) 2019 (Kerkrade, The Netherlands): Dietary intake of advanced glycation endproducts is associated with higher levels of advanced glycation endproducts in plasma and urine – current knowledge and future directions.

Papendal course Vascular Biology 2018 (Papendal, The Netherlands): Restriction of dietary AGEs to prevent diabetes in overweight individuals: design of a randomized controlled trial- deAGEing trial.

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Awards

International Maillard Reaction Society conference (iMARS) 2021: best oral presentation award.

Curriculum Vitae

Armand Linkens werd geboren op 19 april 1993 in Maastricht. Zijn middelbare schooldiploma werd behaald in Maastricht aan het Sint Maartenscollege in 2011. Armand is daarna begonnen aan de bachelor biomedische wetenschappen aan de Universiteit van Maastricht (UM). Na het behalen van zijn bachelordiploma in 2014 werd Armand toegelaten tot de master opleiding Arts-Klinisch Onderzoeker (A-KO), eveneens aan de Universiteit van Maastricht. Zijn aspiratie om dokter te worden werd toen werkelijkheid, nu versterkt door een wetenschappelijke achtergrond. Zijn masterdiploma arts en klinisch onderzoeker behaalde hij in 2018. Uit deze stage vloeide een promotietraject voort onder begeleiding van professor Schalkwijk, professor Stehouwer, dr. Houben en dr. Eussen aan de afdeling interne geneeskunde van de UM. Het promotieonderzoek richtte zich op de gezondheidseffecten van versuikerde eiwitten in voeding, en specifiek het risico op het ontwikkelen van suikerziekte. Na afronding van zijn promotietraject verhuisde hij in 2021 naar Leiden, waar Armand zijn klinische carrière is gestart als ANIOS ouderengeneeskunde binnen Marente. In 2023 hoopt hij zijn carrière te kunnen gaan vervolgen als huisarts in opleiding.

Armand Linkens was born on April 19th 1993 in Maastricht. He graduated from secundary school at the Sint Maartenscollege in 2011. From there, Armand enrolled in the Bachelor study Biomedical Sciences at the University of Maastricht. After receiving his bachelors degree in 2014, Armand was accepted for the Master study Physician-Clinical Researcher (A-KO), also at the University of Maastricht. Armand's aspiration to pursue a medical career finally became reality, now strenghtened by a scientific background. His masters degree as a physican and clinical researcher was obtained in 2018, after which he started working as a PhD-student under the supervision of prof. dr. Casper G. Schalkwijk, prof. dr. Coen D.A. Stehouwer, dr. Boy J.H.M. Houben, and dr. Simone J.M.P. Eussen at the department of Internal Medicine at the University of Maastricht. His PhD-thesis focused on the potential biological effects of dietary AGEs, and specifically the risk of developing type 2 diabetes. Upon completion, Armand moved to Leiden in 2021. Here, he started his medical carreer as a physician in a nursing home. In 2023, Armand aims to continue his carreer as a general practioner.

dAGEs in TOAST
(white bread, 2½ minutes in toaster)
CML: 0.5; CEL: 0.6; MG-H1: 4.2 mg/100 g