The analysis of advanced glycation endproducts

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ADVANCED GLYCATION ENDPRODUCTS
A mass spectrometry-based approach & its applications

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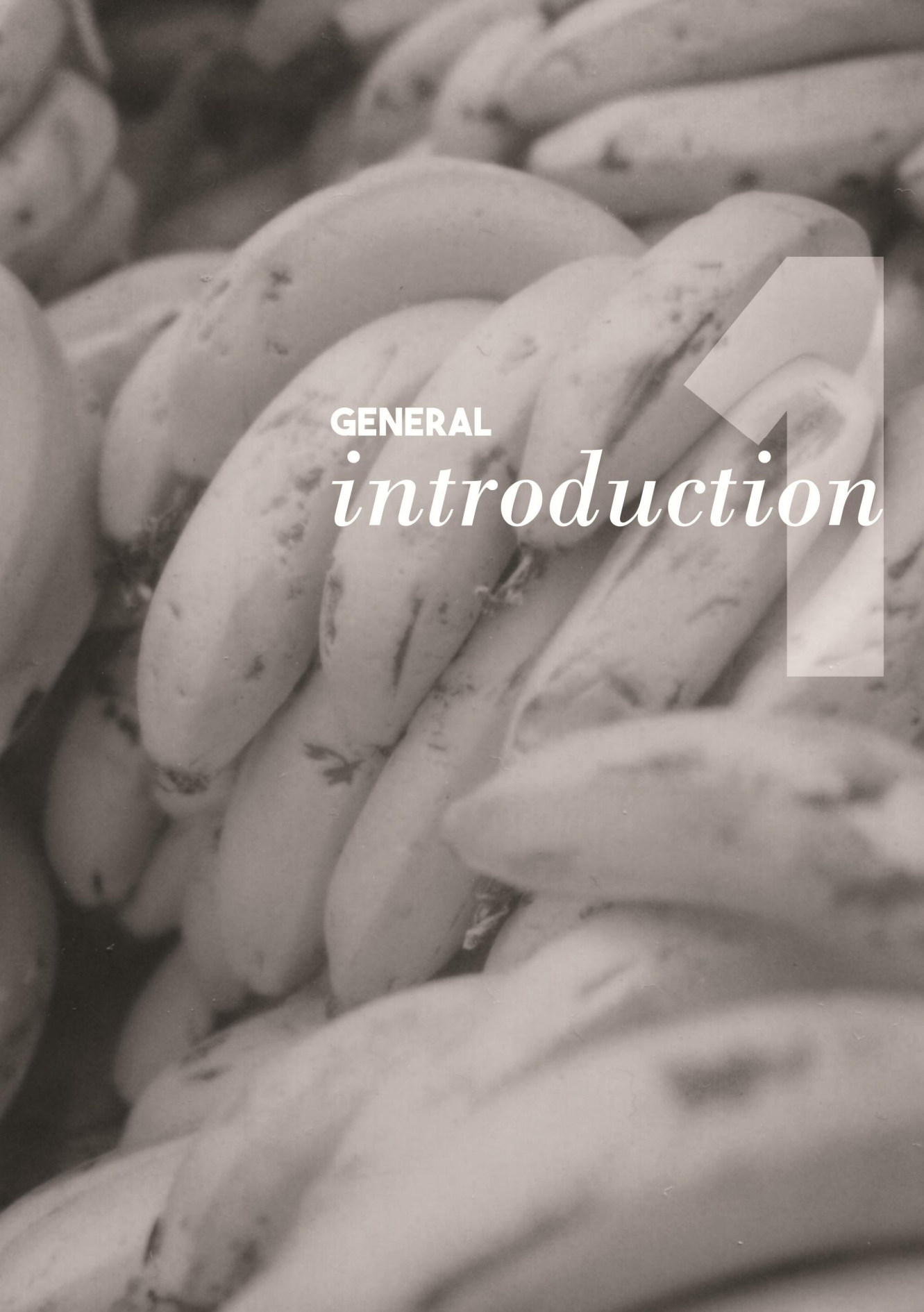
CONTENTS

CONTENTS



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GENERAL
introduction

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General introduction

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General Introduction

Diabetes, vascular disease and advanced glycation endproducts

Age-related, non-communicable chronic inflammatory diseases like cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) represent a major 21st century health problem. The prevalence of these diseases is exponentially rising as the population ages.
Diabetes is a common metabolic disease affecting approximately 422 million people worldwide in 2014[1], which is predicted to rise to 600 million in 2040. In the Netherlands, one million people have diabetes, which is predicted to rise to 1.3 million in 2025[2]. Diabetes is a serious chronic disease characterized by elevated plasma glucose levels. There are two main types of diabetes: type 1 diabetes mellitus (T1DM) and T2DM. T1DM is characterized by an absolute insulin deficiency as caused by an autoimmune destruction of the pancreatic beta cells. T1DM accounts for approximately 10% of the prevalence of diabetes [3]. T2DM is characterized by insulin resistance despite of high levels of insulin. This will ultimately result in an absolute defective insulin secretion. T2DM accounts for approximately 90% of all individuals with diabetes and is primarily caused by lifestyle factors, such as overweight and lack of physical activity[3]. The current obesity epidemic has led to a strong increase in the prevalence of T2DM[4]. The burden of T2DM is mainly determined by macro- and microvascular diseases. Macrovascular disease affects larger blood vessels through atherosclerosis and affects the coronary, the carotid and the peripheral arteries, thus increasing the risk for heart attack, stroke and diabetic foot. Individuals with diabetes are at a 2- to 4-fold increased risk of cardiovascular events compared to non-diabetic individuals[5,6]. CVDs are the number 1 cause of death globally, an estimated 17.5 million deaths were directly caused by CVD in 2012[7]. Microvascular disease affects smaller blood vessels of the eyes, kidneys, and nerves[8] increasing the risk of retinopathy, nephropathy and neuropathy, respectively [9]. Diabetes and its associated complications leads to a permanent and significant loss of quality of life and are acknowledged to be among the highest burden for health care costs[10]. Therefore, prevention of diabetes and in case of diabetes, prevention of cardiovascular disease is of highest importance and clearly represents a medical and economical need. Future strategies should therefore focus primarily on the prevention of diabetes and improved stratification algorithms to allow identification of those who are at risk for developing vascular complications. Early identification of vascular risk is a cornerstone of diabetes management and facilitates tailored intervention at an early stage of disease when a useful response is more likely to be obtained. Given the enormous impact of vascular disease, it is of utmost importance to find biomarkers for the identification of diabetic individuals at high risk of developing vascular complications and morbidity; i.e. to improve risk prediction[11]. So far, the established cardiovascular risk factors and the derived algorithms do not fully explain vascular risk[12]. It is very likely that markers of pathophysiological pathways, which are not covered by the well-known risk factors, will improve risk score.

Various mechanisms have been proposed to explain how hyperglycaemia directly causes diabetic vascular complications, including the polyol pathway, an increase in glucosamine-6-phosphate via the hexosamine pathway, the activation of protein kinase C (PKC) via de novo synthesis of diacylglycerol (DAG) and the non-enzymatic glycation of proteins[13]. The biochemical process of the formation of advanced glycation endproducts (AGEs), which is accelerated in patients with diabetes as a result of elevated plasma glucose levels and increased oxidative stress, has been related to diabetic complications[8,13]. Increased formation and accumulation of AGEs is common in patients with diabetes, and, because of impaired clearance, in particular those with chronic kidney disease (CKD)[14] or end stage renal disease (ESRD)[15]. The group of AGEs is a heterogeneous family of unavoidable by-products which are formed by the reaction of proteins with reactive metabolic intermediates derived from glucose and from lipid oxidation with the involvement of oxidative stress[16-18]. AGEs manifest pathological effects which are implicated in the development of age-related disease[9,19,20]. In addition to the overwhelming amount of data demonstrating a role of AGEs in the development of vascular disease[9,21-25] in individuals with or without diabetes, AGEs are also implicated in the development of insulin resistance and thus the development of diabetes[26]. In addition to endogenous formation of AGEs, it has now become apparent that dietary AGEs represent a significant source of circulating and tissue AGEs[27].

Thus, increased accumulation of AGEs is a core defect, derived from abnormalities in glucose- and lipid-metabolism, impaired clearance or intestinal absorption, which can lead to diabetes and to vascular complications.

However, so far our knowledge about the putative role of specific AGEs in several diseases is hampered by the lack of good analytical techniques to detect these specific AGEs. In addition, the measurement of AGEs may also help to identify patients with a high risk for poor outcome and may thus help in risk stratification[28,29]. Therefore, the central aim of this thesis is the development and validation of state-of-the-art techniques to quantify precursors of AGEs, AGEs and related compounds and the application of these techniques in biochemical research.

Advanced glycation endproducts and related compounds

The non-enzymatic reaction of sugars with proteins, better known as the Maillard or browning reaction, leads to formation of AGEs[17,30]. This reaction was described for the first time in 1912 by the chemist Louis Camille Maillard (Figure 1.1), who demonstrated that amino acids heated in the presence of reducing sugars developed a characteristic yellow-brown color [31]. The classical pathway of the Maillard reaction begins with the condensation reaction of the carbonyl group of sugar aldehydes with the N-terminus or free-amino groups of proteins to the formation of a reversible Shiff base (Figure 1.2). This labile adduct undergo rearrangements, through acid-base catalysis, leading to the formation of the more stable Amadori products. These glycation adducts are classified as early glycation products.

The chemistry of these early glycation products has been well described, with glycated hemoglobin (HbA1c) as the best-studied example[32]. Only a small part of these stable, but still reversible formed early glycation products, undergo further oxidative reactions to form a variety of irreversible AGEs[33]. One major consequence of the advanced Maillard reaction is the formation of covalently cross-linked proteins which, in long-lived tissues (e.g. skin collagen and lens proteins), accumulate with age[18,34,35]. So far, one of the best characterized lysine-arginine cross-link is the fluorophore pentosidine[35,36].

Figure 1.1 Louis Camille Maillard (1878-1936) photographed in his laboratory around 1915.

In addition to the classical Maillard reaction we now know that *in vivo* AGEs are also formed rapidly from highly reactive intermediate dicarbonyl groups, known as oxoaldehydes (or dicarbonyls), including methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG) (Figure 1.3)[13]. Accumulation of these reactive oxoaldehydes is known as “dicarbonyl stress”. Many different sources are responsible for the formation of these dicarbonyl metabolites, in particular by increased glucose metabolism but also by lipid peroxidation, degradation of glycated proteins and by intestinal absorption[37]. MGO, the most potent glycation agent, is formed by the non-enzymatic fragmentation of the triose phosphates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, which are derived from glucose and fructose metabolism.

This intracellular dicarbonyl compound reacts mainly with arginine to form N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1, MG-H2 and MG-H3)[38,39] and with lysine to form N^{ε}-(1-carboxyethyl)lysine (CEL). MGO is detoxified by the glyoxalase pathway by two dependent thiol-enzymes; glyoxalase-1 and -2 (GLO-I and GLO-II) and reduced glutathione (GSH) into D-lactate (Figure 1.3)[40,41]. Glyoxal is formed by the degradation of glucose by retro-aldol condensation reactions[42] activated by deprotonation of the 2- and 3-hydroxy groups[43]. Glyoxal reacts further with lysine to form N^{ε}-(carboxymethyl)lysine (CML), a well-known ligand for the receptor for AGEs (RAGE) [44]. The reduction of glucose to sorbitol and further oxidation to fructose and fructose-3-phosphate leads to the formation of 3-DG. Thus, AGEs can be formed by these sugar-derived carbonyls[45], by lipid peroxidation and oxidative stress[46,47] or by reactions between pentoses and proteins, known as the fluorescent lysine-arginine cross-link pentosidine[36,48].

AGEs, in our body, can also originate from exogenous sources such as diet and smoking[49,50]. AGEs in foods are formed rapidly during processing at high temperatures, such as grilling, frying, baking or roasting[49,51,52]. Since the consumption of these processed foods has increased over the past decades[53], the exposure to dietary AGEs has also increased and may contribute to the development of chronic diseases[27,54,55].

It was not until 1980 that the pathophysiological significance of AGEs emerged in medical science[17,30]. So far, accumulation of AGEs in the body, is implicated in the pathogenesis of age-related diseases in particular in relation to diabetes, inflammation, neuro-degenerative disorders and cardiovascular disease[21,56-59]. Several mechanisms have been proposed by which AGEs may adversely affect human health; including AGE accumulation in the extracellular matrix proteins, leading to the formation of cross-links and subsequently vascular stiffening, the binding of AGEs to AGE-receptors, consequently activating NFκB and MAP-kinase signaling pathways, and cellular dysfunction as a consequence of altered function of proteins by intracellular glycation[13,60,61].

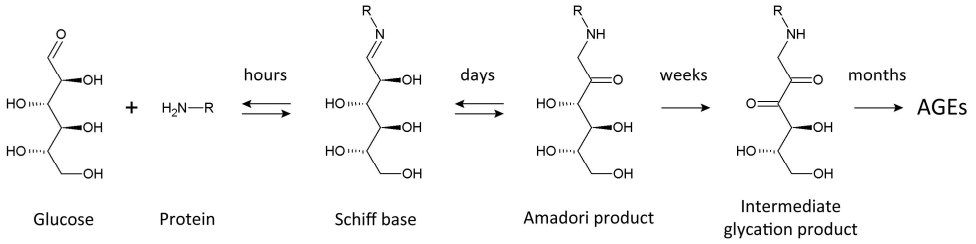

Figure 1.2 Scheme of the formation of AGEs by the Maillard reaction.

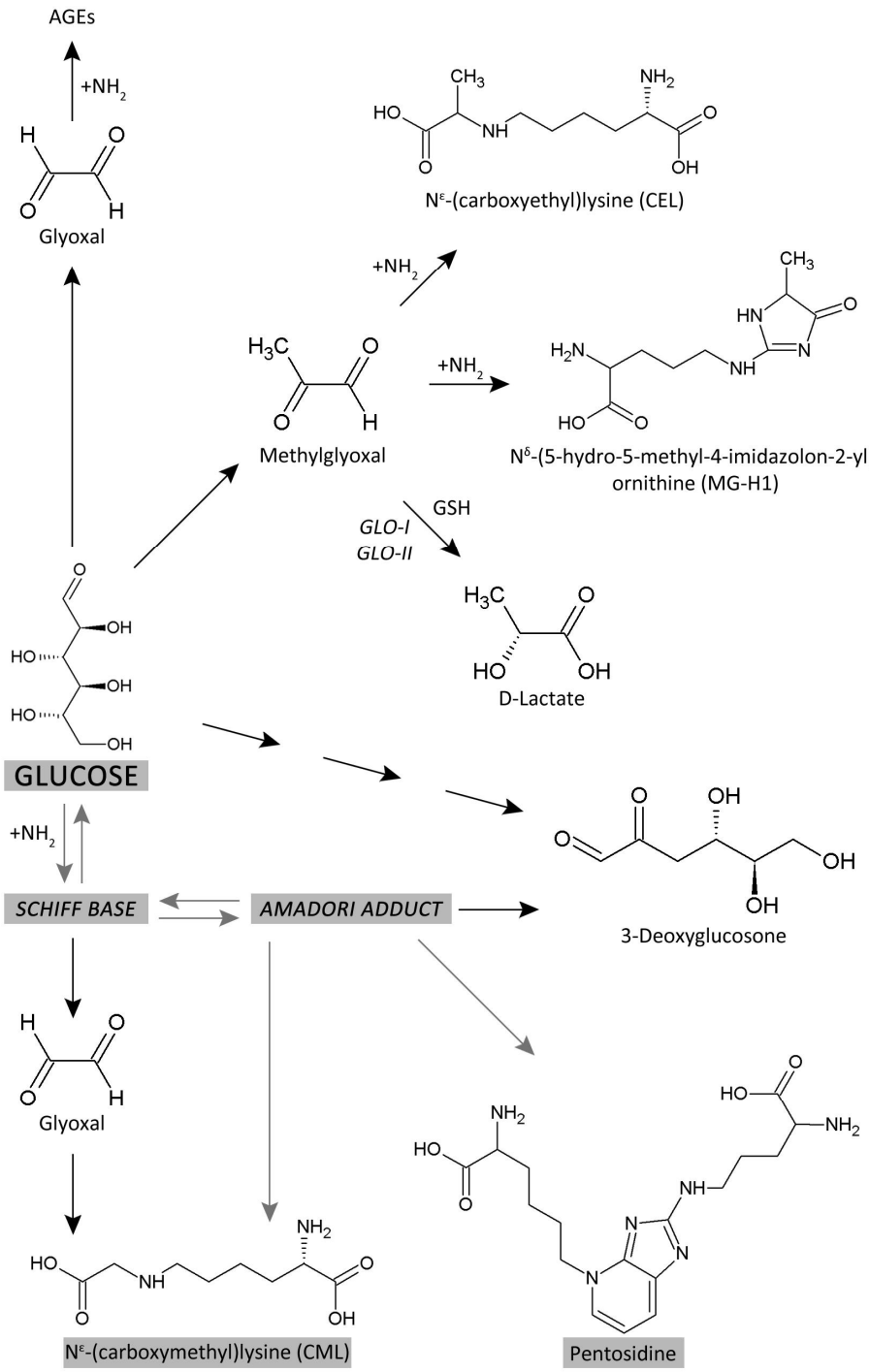

Figure 1.3 The formation of advanced glycation endproducts.

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Analysis of advanced glycation endproducts

The early beginning of AGE analysis: fluorescence detection

Since the discovery of AGEs in 1912, by Louis Camille Maillard, it took almost 70 years until the first *in vivo* analyses were done[17,30,62]. In those days, AGEs were detected as a group of advanced glycation products (AGP) with a brown or fluorescent property. Insoluble human dura mater collagen was digested with collagenase and the absorbance at 350 nm and fluorescence at 370/440 nm (excitation/emission) of the solubilized material was measured[62]. For the first time AGE accumulation was estimated in long-lived proteins, such as collagen and lens crystallins, and this was closely related to the pathogenesis of aging and diabetes[17,30,62]. More recent, skin autofluorescence (SAF)[63] is used as a non-invasive tool to assess the AGE accumulation in the skin and can be useful for rapid risk assessment of AGE-related long-term complications in diabetes and related diseases[64-67]. SAF illuminates approximately 1 cm^2 skin with an excitation light source of 300 to 420 nm. Autofluorescence was calculated by dividing the average light intensity emitted per nm over the 420 to 600 nm range by the average light intensity emitted per nm over the 300 to 420 nm range[63]. The specificity of these fluorescence techniques is limited because they cannot quantify the concentrations of individual compounds and major AGEs are not fluorescent (e.g. MG-H1, CML and CEL). Moreover, interference of other fluorophores, e.g. nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), porphyrins, dityrosine and N'-formylkynurenine[68-70], or changes in the absorption of ultraviolet (UV) excitation light due to hemodynamic and oximetric effects[71] may influence the relationship between (auto)fluorescence and AGE. Therefore, the interpretation of SAF data, as an estimate of AGEs, and the relationship with disease should be interpreted with care. Nevertheless, SAF can be used as a marker of the total skin AGE pool and was linked to age, renal failure and is closely related to risk-factors for long-term diabetic complications[66,67,72].

The next step in AGE analysis: immunochemical detection

To get a better understanding of the pathological consequences of AGEs a more specific technique was needed. In the beginning of 1990, Nakayama[73], Makita[74], Horiuchi[75] and co-workers described immunochemical approaches using antibodies specific for AGEs on proteins. Mono- and polyclonal anti- antibodies against AGE-epitopes were raised and AGE measurements were performed with an enzyme-linked immunosorbent assay (ELISA). Although immunoassays can be applied as a high throughput, simple and cheap method to quantify AGEs, it remained difficult to produce reliable and reproducible results. In the following years ELISA-based techniques were improved and several antibodies against mainly CML-modified proteins were developed and used for AGE analysis[76-80]. However, quantification of AGEs with ELISA-based techniques did not give satisfying results. The antibody 6D12, which was widely applied to quantify CML, showed cross-reaction to CEL, thus indicating the lack of epitope specificity[81,82].

Moreover, the use of blocking proteins, such as serum albumin and gelatin, to decrease the non-specific binding of primary and secondary antibodies, gave misleading results because of the presence of glycated amino acids in these blocking proteins[83]. Also, pre-analytic processing such as the use of extreme high pH or heating, degrade N^{ϵ}–fructosyl-lysine residues rapidly to form AGEs[84,85]. Indeed, most recent papers on the comparison of ELISA-based techniques with instrumental techniques, such as gas chromatography mass spectrometry (GC-MS)[86,87] and liquid chromatography tandem mass spectrometry (LC-MS/MS)[87,88], revealed inconsistent results. We have compared the CML content in food items, as analyzed with our UPLC-MS/MS technique with results from an ELISA based technique, as described by Uribarri et al.[89] and with a UPLC-MS/MS technique, as reported by Hull et al.[90] (Figure 1.4). In 29 food items, based on the same description and preparation of each food item, we have measured the CML content and found no significant correlation between our UPLC-MS/MS and the ELISA technique (r=0.32, N.S, p=0.74). However, a significant correlation was found between both UPLC-MS/MS techniques (r=0.82, p<0.0001) (Figure 1.4).

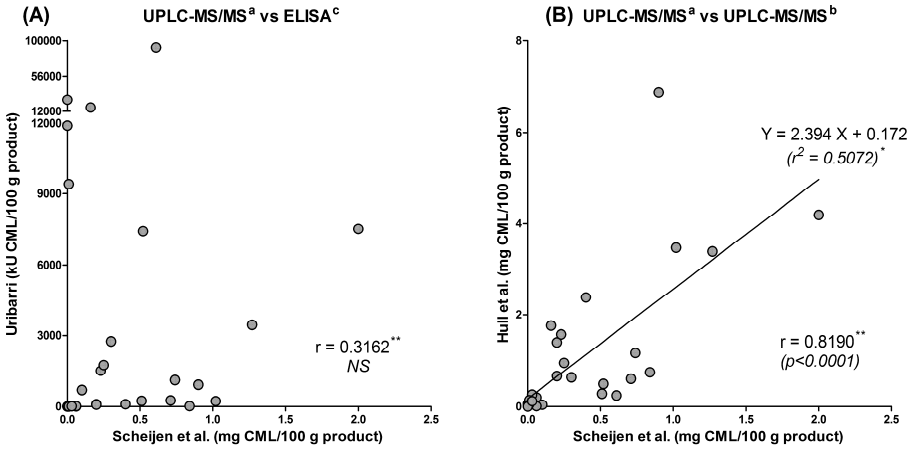

Figure 1.4. Comparison of CML content in selected food items analyzed with different techniques.
(A) Correlation of CML content in 29 food items as analyzed with our UPLC-MS/MS technique and ELISA described by Uribarri et al. (Uribarri et al., 2010)
(B) Correlation of CML content in 29 food items as analyzed with our UPLC-MS/MS technique and the UPLC-MS/MS technique described by Hull et al. (George L.J. Hull et al., 2012)
(* r-square based on linear regression, ** r based on Spearman correlation, NS = not significant (p>0.05)).
[a]CML content in food items based on UPLC-MS/MS analysis by Scheijen et al.
[b]CML content in food items based on UPLC-MS/MS analysis by Hull et al. (George L.J. Hull, 2012)
[c]CML content in food items based on ELISA analysis by Uribarri et al. (Uribarri et al., 2010)

Though, an advantage of specific antibodies is the use of immunohistochemistry to stain tissues, such as brain tissue[91], atherosclerotic lesions[92] and aortic tissues[93]. This technique is widely used to study the distribution and localization of specific AGEs in biological tissues. It is a simple, highly sensitive, specific and semi-quantitative technique for the detection of AGEs in many different tissues. Thus, many estimates of AGEs were done with immunochemical methods but numerous problems have arisen and therefore AGE analysis on a molecular level with analytical techniques is required.

1

INTERMEZZO

History of chromatography

In 1903, chromatography was invented by the Russian botanist Mikhail Semyonovich Tsvet [94-96] and coined the word chromatography which is coming from the Greek word chroma meaning color and graphein meaning writing [95]. He used column chromatography to study pigments in plants and one of his first methods was published in 1906 [97]. It took until the year 1930 before Tsvet's work found his attention in the scientific world. In 1931, Kuhn, Winterstein and Lederer published their method to study carotene-like compounds and Reichstein used the same technique to isolate corticosteroids [94]. Liquid chromatography, as we know it today, was used for the first time in 1941 by Archer John Porter Martin and Richard Laurence Millington Synge [98]. They used liquid-liquid extraction chromatography to study the amino acid composition of wool. In 1952, the Nobel Prize in Chemistry was awarded jointly to Martin and Synge for their invention of partition chromatography [99]. Further development of liquid chromatography to the modern and general purpose high performance liquid chromatography (HPLC) system was done at the early 1960s by Csaba Horvát and Josef Huber [100]. It was in the late 1960s that firms like Waters Associates and DuPont introduced commercial HPLC equipment and this leaded to the first HPLC paper in 1966 [101]. The number of HPLC-related publications grew from that year on exponentially [100].

Ultra performance liquid chromatography

Since the introduction of the general purpose HPLC system, many manufacturers worked on the improvement of different parts, such as better pumps, injectors and detectors of the HPLC system. Besides these developments there were other fundamental improvements needed to enable HPLC to become a leading analytical technique. The evolution of the packing materials, particularly the decrease of the particle size, improved column efficiency to new limits [102].

To investigate column efficiency, van Deemter stated an empirical formula describing the relationship between linear velocity (u, mm/s) and plate height (H, µm), the so-called van Deemter H-u curve (Figure 1.5) [103]. With this equation it was possible to compare column performance between columns packed with different particle size. Small plate heights indicate higher efficiency and subsequently narrow peaks. As from the introduction of HPLC in the late 1960s, column particles decreased from 10 µm in the 1970s, to 5 µm in the 1980s, to 3.5 µm in the 1990s and to 1.7 µm in 2004[102]. By using this smaller particle size, a significant gain in efficiency was reached (Figure 1.5). Not only faster chromatography was achieved but also better resolution and increased sensitivity, due to sharper and higher peaks[104]. With the introduction of 1.7 µm particles column efficiency was no longer dependent on flow rate or linear velocity (Figure 1.5)

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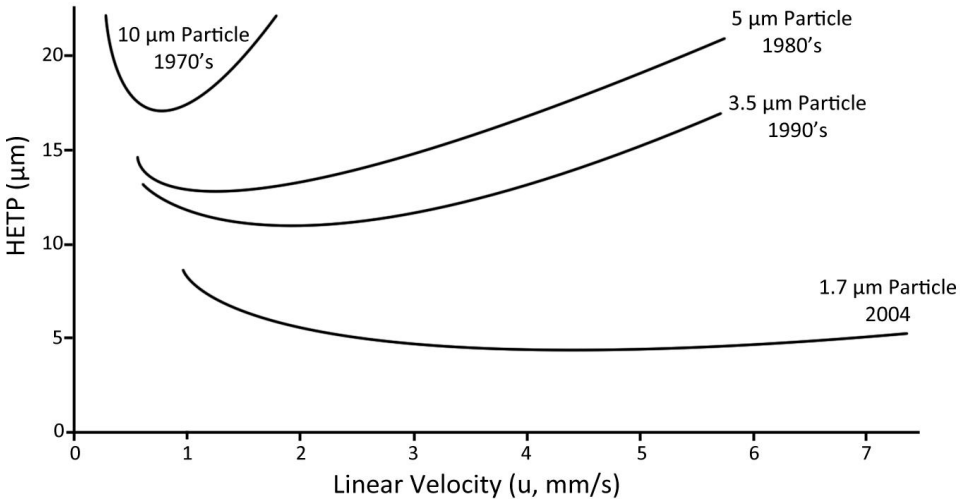

Figure 1.5 Van Deemter H-u curve, evolution of particle size and column efficiency.

High sensitive and specific detection of biomolecules

Since the introduction of HPLC and UPLC in biomolecule analysis, a highly specific and sensitive detection method was needed. Mass spectrometry is one of these detection techniques, used for the first time in 1897 by Sir Joseph John Thomson[105] and further expanded by Wolfgang Paul[106], Malcolm Dole[107] and John Benett Fenn[108] and led to more than 7700 methodological publications in 2016 (figure 1.6).

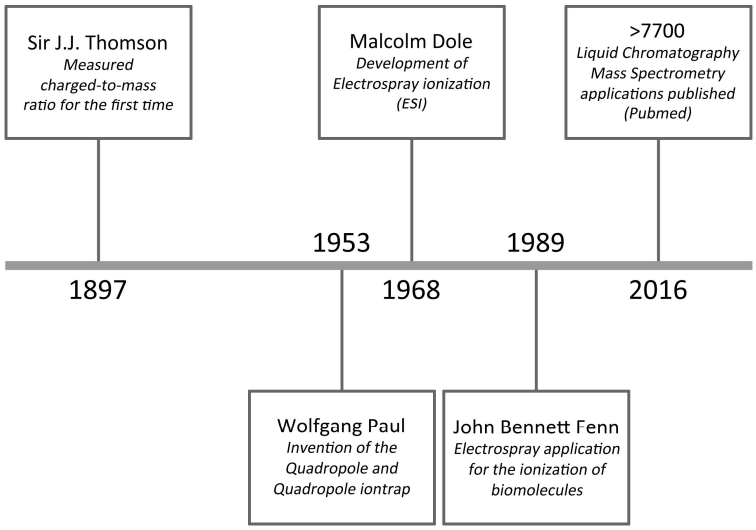

Figure 1.6 Important 20th-century developments in liquid chromatography mass spectrometry.

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From the 1990s onwards, several key developments have made MS one of the strongest tools in biomarker quantification and discovery. Tandem mass spectrometry (MS/MS) was one of these important developments, introduced in 1968 by Professor Keith R. Jennings and Professor Fred McLafferty[109]. A tandem MS, also known as a triple quadrupole MS, consists of two MS stages with a fragmentation cell in between the two stages (figure 1.7). Ions are formed in the ion source and one specific ion (precursor or parent ion) is selected by the first stage (MS1). Next, this parent ion is fragmented by collision induced dissociation with argon atoms creating fragment ions (daughter ions) which are selected in the second stage of the MS (MS2) (figure 1.7).

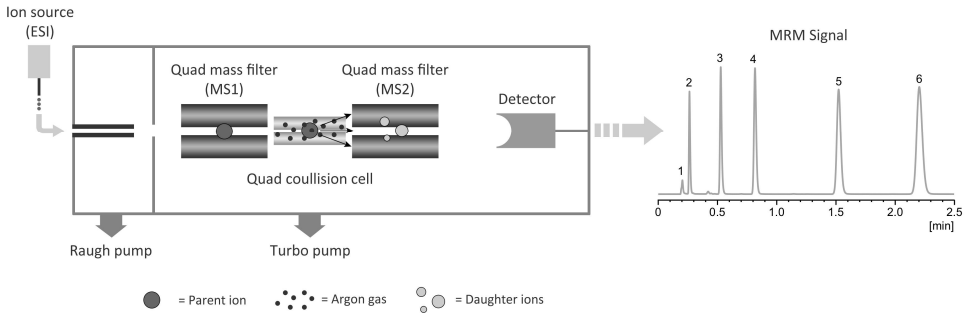

Figure 1.7 Schematic view Tandem mass spectrometer.

By selecting the most abundant fragment ion a multiple reaction monitoring (MRM) transition is made and in this way a highly specific detection is possible. Each compound, including its corresponding stable isotope, is characterized by its own chromatographic retention time, parent ion (m/z) and daughter ion (m/z). Quantification of the compounds of interest is performed by calculating the peak area ratio of each unlabeled peak area to the corresponding internal standard (stable isotope) peak area.
UPLC-MS/MS analysis provides reliable and robust quantitative data with very small amounts of sample (varying from 20 to 30 µL plasma or urine) and is applicable for a broad range of biomolecules. Application of a fully-programmable auto-injector makes it possible to analyze >100 samples per day and is therefore very useful for studying disease in large cohort studies. From 1981 until 2015 an exponential increase in LC-MS/MS publications has been seen and underlines the significance of this technique to quantify a broad range of analytes (figure 1.8).

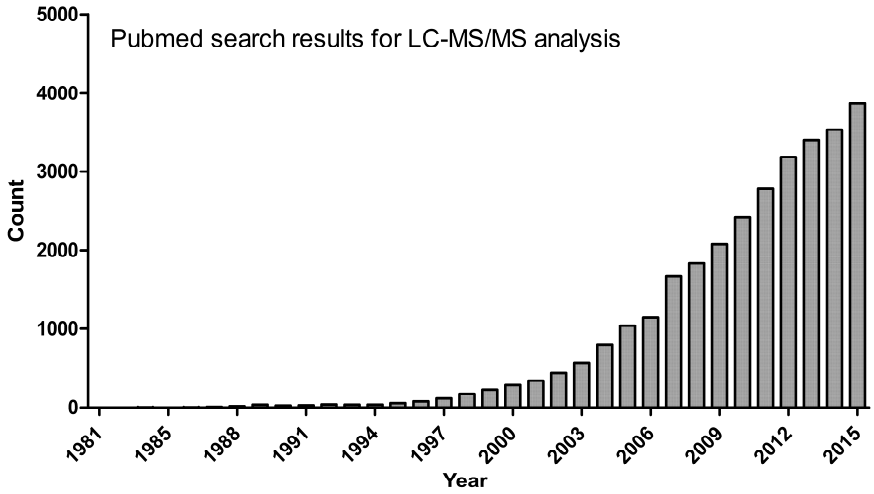

Figure 1.8 Increase of LC-MS/MS applications as published over the years.

The gold standard in AGE analysis: mass spectrometry detection

Quantification of AGEs with instrumental methods, such as liquid chromatography (LC) or gas chromatography (GC), needs a completely different sample preparation compared to immunochemical techniques. Free (modified) amino acids are needed in case of the instrumental methods whereas intact (glycated) proteins can be used for immunochemical techniques. To obtain these free (modified) amino acids, the peptide bonds of the intact protein and peptides have to be hydrolyzed. Two hydrolyzation methods have been described, namely by hydrochloric acid[88,110] and by enzymatic digestion[39,111]. Acid hydrolysis is performed by heating the protein (pellet) with 6 mol/L hydrochloric acid for 24 hours at a temperature of 110°C in sealed vials. This way of hydrolyzation is cheap, easy and is the most reliable procedure to obtain free amino acids. Prior to this hydrolysis it is necessary to perform a reduction step with sodium borohydride to prevent formation of CML out of early glycation products during heating in hydrochloric acid[112]. However, some AGEs, such as hydroimidazolones and pyrralines are acid unstable, and therefore enzymatic hydrolysis has been used[113]. For the compounds that are acid unstable and could not be adjusted for the decrease after acid hydrolysis, as we have demonstrated for MG-H1 (Chapter 2B), enzymatic hydrolysis is an alternative. Enzymatic hydrolysis is carried out using a cocktail of endo- and exopeptidases, which cleaves the peptide bonds within and at the end of the protein, respectively. A mixture of pepsin, pronase E, prolidase and aminopeptidase was used for many different purposes[113-115]. However, the recovery of the release of free amino acids by this enzymatic hydrolysis remains questionable. Due to blockage of the cleavage sites by modified arginine and lysine residues, inhibition of the protease by some glycation adducts and lack of cleavage due to side chain modifications, enzymatic hydrolysis may lead to an underestimation of AGE content[116,117]. Therefore, in case of acid stable analysis of AGEs, the most preferable hydrolyzation technique is acid hydrolyzation.

Acid hydrolyzation has been widely used to hydrolyze plasma and food matrices to quantify AGEs with instrumental methods, including GC-MS[86,118] and LC-MS[52,110,119]. Purification and separation of AGEs from the broad range of other polar compounds, in plasma and food sample hydrolysates, is of utmost importance before detecting them with these MS techniques. In particular, avoiding quenching of the compounds of interest, when using electrospray ionization (ESI) is challenging[120,121]. With the introduction of ultra-performance liquid chromatography (UPLC) in 2004, a significant gain in resolution and thus separation of complex matrices was achieved[102]. The use of UPLC in combination with stable-isotope dilution and subsequently detection with ESI multiple reaction monitoring (MRM) is the best available analytical technique to quantify AGEs.
Besides the use of a state-of-the-art technique, such as UPLC-MS/MS, to quantify AGEs and other biomolecules, derivatization of the compounds of interest could increase sensitivity and selectivity. A broad range of derivatization reagents have therefore been used to detect biomolecules with MS techniques, including 1-butanol to derivatize amino acids[122], diacetyl-L-tartaric anhydride (DATAN) to derivatize glutaric acids[123] and O-phenlylenediamine (oPD) to derivatize α-oxoaldehydes[124]. These three reagents have been used in this thesis to separate and quantify butylated AGEs, enantiomeric L- and D-lactate and α-oxoaldehydes as quinoxaline derivatives with ESI-UPLC-MS/MS. AGEs and other related compounds were detected and quantified by stable isotope dilution UPLC-MS/MS. Plasma, urine or food samples were prepared according to the physical properties of the compound of interest and sample matrix. Deproteinization and subsequently derivatization of the dried supernatant, in case of the free adducts, and hydrolyzation of the protein-pellet and subsequently derivatisation of the dried hydrolysate, in case of the protein-bound adducts, was applied before analysis.
Derivatives, and their corresponding stable isotopes, were first separated with UPLC and then ionized with ESI-positive mode and detected by MRM.

Aims and outline of this thesis

Specific, sensitive and reliable techniques are needed to quantify a broad spectrum of well-characterized AGEs. Since the introduction of HPLC in the late 1960s and further development to UPLC in 2004 this state-of-the-art technique came available. The aim of this thesis is to develop and validate several liquid chromatography techniques for the detection of precursors of AGEs and AGEs as well as an application of each technique.

Chapter 2A describes a rapid and sensitive single-column HPLC method for the quantification of protein-bound pentosidine with fluorescence detection. With this technique, we have measured plasma levels of protein-bound pentosidine in uremic patients on peritoneal dialysis and compared to healthy controls.

Chapter 2B describes a sensitive, simple and highly selective method to determine protein-bound and free CML, CEL and MG-H1 in plasma by UPLC-tandem MS. Plasma free and protein-bound AGEs were measured in healthy volunteers and patients on peritoneal dialysis.

In Chapter 3, the association of the above mentioned plasma AGEs with prevalent CVD was investigated in individuals with various degrees of glucose metabolism. Plasma levels of AGEs were quantified using both methods as described in Chapter 2A and B.

D-lactate is the degradation product of methylglyoxal by the glyoxalase pathway. Therefore, D-lactate can be used as a reflection of methylglyoxal. In Chapter 4, the quantification of D- and L-lactate using UPLC-tandem MS without the use of chiral stationary phase chromatography was described. D- and L-lactate were analyzed in plasma and urine of controls, patients with inflammatory bowel disease and patients with T2DM.

In Chapter 5, the association between plasma D-lactate and insulin resistance was investigated.

Chapter 6 describes a method for the quantification of the α-oxoaldehydes; methylglyoxal, glyoxal and 3-deoxyglucosone with use of UPLC-tandem MS. An improved sample preparation is evaluated and increased plasma levels of T2DM patients were found as compared to non-diabetic controls.

In Chapter 7, plasma levels of α-oxoaldehydes were investigated after a glucose load in individuals without and with impaired glucose metabolism and T2DM patients.

Chapter 8 describes a validation of an UPLC-tandem MS method for the determination of AGEs in food items and presents a database of dietary AGEs in a selection of food items commonly consumed in a Western diet.

In Chapter 9, the association of dietary AGEs with plasma and urinary AGEs was investigated.

The results of this thesis are summarized and discussed in Chapter 10.

1

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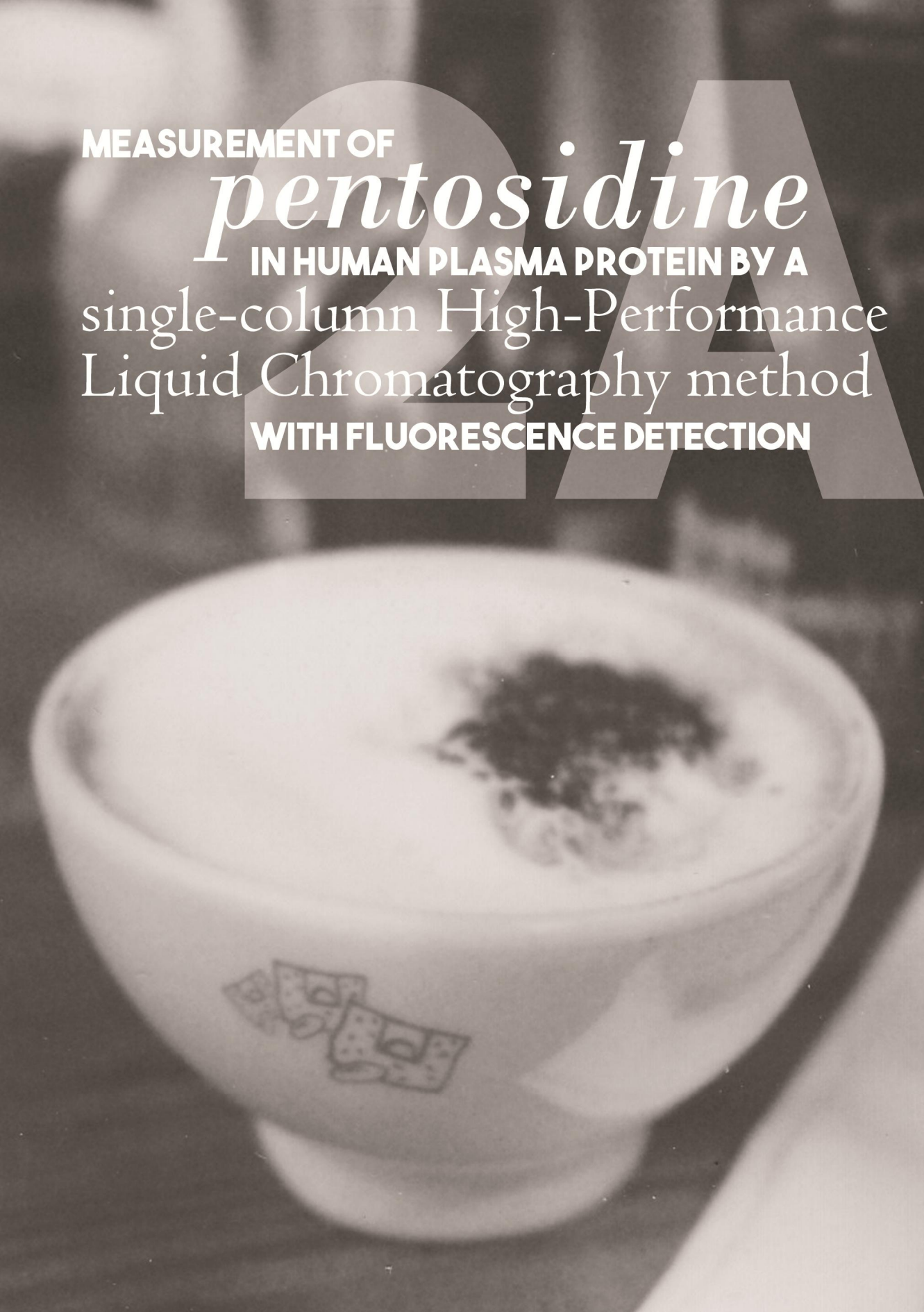

2A

MEASUREMENT OF *pentosidine* IN HUMAN PLASMA PROTEIN BY A single-column High-Performance Liquid Chromatography method WITH FLUORESCENCE DETECTION

2A

Measurement of pentosidine in human plasma protein by a single-column High-Performance Liquid Chromatography method with fluorescence detection

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Abstract

A rapid and sensitive single-column High-Performance Liquid Chromatography method and application for the detection of protein bound pentosidine is described. Pentosidine, a cross-link between arginine and lysine, is a well-characterized advanced glycation endproduct. In order to detect protein-bound pentosidine, plasma proteins were hydrolysed in 6 N HCL. Detection of pentosidine is done based on its own fluorescence characteristics using fluorimetric detection (Ex=325 nm, Em=385 nm). Separation is done, with a run-to-run time of 30 minutes, on a C18 Allspehere ODS-II column with a citric acid acetonitril buffer. This detection enables sensitive and specific determination of protein bound pentosidine in plasma with a detection limit of 2.2 nmol/L or 0.02 pmol/mg protein (signal-to-noise: 6). The intra-assay coefficient variation is 6.5% at a plasma pentosidine concentration of 0.47 pmol/mg protein and 2.0% at a concentration of 1.27 pmol/mg protein. The inter-assay coefficient variation is 3.1% at a plasma pentosidine concentration of 0.43 pmol/mg protein and 1.6% at a concentration of 1.40 pmol/mg protein. Linearity is tested in 4 different plasma samples and showed linearity (0-200 nmol/L, r2>0.99). Recovery of pentosidine in 4 different plasma samples at different concentration levels is 102±10% (mean±SD). Using this method protein bound pentosidine concentration is investigated in healthy controls (n=24, age 67±9 years) and patients with end stage renal disease (n=24, age 65±10 years). Higher plasma concentrations of protein bound pentosidine are measured in the patient group as compared with the control group 3.05 (2.03-3.92) pmol/mg protein and 0.21 (0.19-0.33) pmol/mg protein, respectively (median (interquartile range), p<0.00001). These results are consistent with previously reported results.

Introduction

Glycation is the nonenzymatic reaction of glucose or other reducing sugars with amino groups of proteins. The amino groups of the side chains of arginine and lysine are the primary targets for this type of posttranslational modification. Over time, the initial glycation products may undergo intramolecular rearrangements and oxidation reactions and ultimately transform into stable, so-called advanced glycation endproducts (AGEs). AGEs represent a mixture of different products such as pentosidine and N^{ε}-(carboxymethyl)lysine (CML). AGEs have different biological functions: some are protein cross-links such as pentosidine and some, such as CML, are recognition factors for specific AGE-binding receptors. The physiological consequences of AGEs in ageing and in the aetiology of diabetic complications[1] and in the development of age-related diseases such as inflammation[2], atherosclerosis[3] and neurodegenerative disorders[4] has been described.

Despite progress in this field, a more comprehensive understanding of the putative effects of AGEs in the pathophysiology of these diseases is needed. In addition, the measurement of AGEs may also help to identify patients with a high risk for poor outcome and may thus help in risk stratification[5,6]. However, progress in the field is hampered by the lack of an easy method for the detection of AGEs in the laboratory. We need a rapid, simple and reliable method for the detection of AGEs.

AGEs have traditionally been detected by enzyme-linked immunosorbent assay (ELISA)[7-9]. For several reasons the use of antisera for quantitative immunoassays of protein-bound AGEs is questionable. Reproducibility and sensitivity of such an assay are not optimal, because the specificity of the antibodies is often difficult to define and, because of steric constraints, not all AGE epitopes on the protein may be available for interaction with the antibody. Thus, AGE measurements with immunoassays should be interpreted with care.

A better approach for the quantitative determination of specific AGEs in proteins is the use of a specific analytical technique. For the determination of AGE levels in both tissue and blood samples, high performance liquid chromatography (HPLC) measurements[10-16] and several mass spectrometry methods have been developed including gas chromatography mass spectrometry (GC–MS)[17], and liquid chromatography tandem mass spectrometry (LC-MSMS)[18,19]. Although the latter is considered to be the most accurate technique available at this moment for the detection of AGEs, a disadvantage is that this method is very expensive and not suitable for routine clinical use.

Pentosidine, one of the best-characterized AGEs, is a fluorescent cross-link between arginine and lysine. Because pentosidine is stable under the conditions used for acid protein hydrolysis and can be detected at very low concentrations based upon its fluorescence properties, pentosidine can be regarded as a biomarker for AGEs. Until now, pentosidine has been quantified by ELISA[7,9], by HPLC[10-16,20-25] and by LC-MSMS[19,28]. In addition to the restrictions to use immunoassays for the detection of AGEs as described above, antibodies against pentosidine or specifc ELISA systems are not commercially available.

Accurate quantification of pentosidine by HPLC could only be obtained with cumbersome double-chromagraphic systems[10,13,14,20], with ion-exchange HPLC with long run-times and high variations in retention-time[21], with RP-HPLC with the use of ion-pairing agent with low recoveries[10,12,26] or HPLC analysis with elaborate solid phase extraction (SPE) sample preparation[16].
We now describe a rapid, simple and sensitive one-column reversed-phase HPLC method for the detection of pentosidine in plasma protein hydrolysates.

Materials and methods

Instrumentation

Samples were analysed by reversed phase HPLC-fluorescence using an Allsphere ODS-2 (150x4.6 mm, 3 µm) analytical column fitted with an Allsphere ODS-2 (7.5x4.6, 5 µm) precolumn (Alltech/Grace, Breda, The Netherlands). Detection was carried out using a Jasco type 821-FP spectrofluorometer (Jasco Benelux, Maarssen, The Netherlands) set at an excitation and emission wavelength of 325 and 385 nm, respectively. HPLC analysis was performed using a binary high pressure gradient at a flow of 1 mL/min using two Model PU-980 pumps (Jasco Benelux, Maarssen, The Netherlands). Solvent A was 25 mmol/L citric acid and solvent B was (50/50 v/v) ACN/25 mmol/L citric acid. A linear gradient was started at 99% solvent A which was changed within 15 minutes to 90% solvent A. After cleaning the column with 100% solvent B during 5 minutes the column was equilibrated for 8 minutes at the initial composition. Injection volume was 10 µL and column temperature was set at 20°C using a Spark Mistral column oven (Spark Holland B.V., Emmen, The Netherlands). Samples where thermostatted at 6°C using an auto-injector model 717 Plus Autosampler (Waters, Etten Leur, The Netherlands). Chromatograms were acquired and processed with Totalchrom (Perkin-Elmer, version 6.2.0.0.0:B27, Zoetermeer, The Netherlands).

Materials

HPLC-grade acetonitril (ACN) was obtained from Chromanorm (Prolabo, Paris, France). Citric acid (GR for analysis), sodium hydroxide and hydrochloric acid (HCl) fuming (37%) were obtained from Merck (Darmstadt, Germany). Trichloroacetic acid, boric acid (99.5%) and trifluoroacetic acid (TFA) (99+%) were obtained from Sigma (SigmaUltra min. 99,0%) (Zwijndrecht, The Netherlands). Ultra pure waters was generated by a Super-Q system (Millipore, Amsterdam, The Netherlands) and sodium borohydride was obtained from Fluka (Buchs, Schweiz). Pentosidine standard was obtained from IMARS (International Maillard Reaction Society, http://imars.case.edu/). Levels determined with this standard are four times lower than published by Sell et al.[27] and agree with Dyer et al.[28].

Plasma samples

Heparinized plasma samples were obtained form 24 healthy volunteers (16 male, 8 female, mean age 67±9 years) and 24 uremic patients on peritoneal dialysis (PD) (14 male, 10 female, mean age 65±10 years).

Sample preparation

In a 10 mL glass tube with a Teflon-lined screw-cap 50 µL plasma was mixed with 100 µL water. To prevent a potential artifactual formation of pentosidine from early glycation products during sample preparation, plasma samples were reduced by 500 µL sodium borohydride borate buffer (200 mmol/L, pH=9.2) before precipitation. This mixture was allowed to stand for 2 hours at room temperature. Proteins were then precipitated by addition of 2 mL 20% trichloroacetic acid and centrifuged for 10 minutes (4°C) at 4500 g. The supernatant was carefully removed by aspiration with a Pasteur pipette. The protein pellet was washed once by adding 2 mL 5% trichloroacetic acid followed by centrifugation and removal of the supernatant as described above (The effect of this extra washing step on absolute peak area and recovery was tested negative). For recovery and linearity experiments 50 µL of pentosidine standard (0-200 nmol/L) was added. Samples were hydrolyzed by adding 50 µL 6N HCl and incubation for 18 hours at 110°C. After hydrolysis, samples were evaporated to dryness at 80°C under a stream of nitrogen gas and reconstituted in 200 µL 25 mmol/L citric acid/L. This solution was centrifuged for 15 minutes (4°C) at 14000 rpm. Ten micro liter of this solution (equals 2.5 µL plasma) was injected on the HPLC system.

Standard pentosidine

Stock standard pentosidine was prepared by dissolving pentosidine in 0.1% (v/v) TFA at a concentration of 20 µM. Standard was divided in portions and stored at -20°C prior to use.

Determination of protein in plasma

Pentosidine concentration was expressed as pmol/mg protein in plasma. Protein concentration in plasma was analysed using Bradford reagent (Biorad Laboratories GMBH, München, Germany)[29]. The calibration curve was established using bovine albumin fraction V (Sigma, Zwijndrecht, The Netherlands).

Statistical Analysis

The method validation data were expressed as mean and SD. The patient study data were expressed as median and interquartile ranges (IQR). To detect group differences we applied the Mann-Whitney U test.

Results

Reversed phase chromatography

During method development we tested first cation-exchange chromatography, as described by Mitsuru Saito et al. 21 and different ion-pair based HPLC systems. However, with these methods we observed retention time variations of >1 min with no baseline separation of pentosidine (data not shown). In contrast, with RP-HPLC, we found a retention time of 13.24±0.07 min (CV 0.5%, n=50 different plasma samples) and a baseline separated pentosidine peak. A chromatogram of a pentosidine standard, a typical chromatogram of a plasma sample from a healthy control and from a uremic patient on peritoneal dialysis are shown in figure 2A.1. The baseline separated pentosidine peak in plasma makes automatic integration possible.

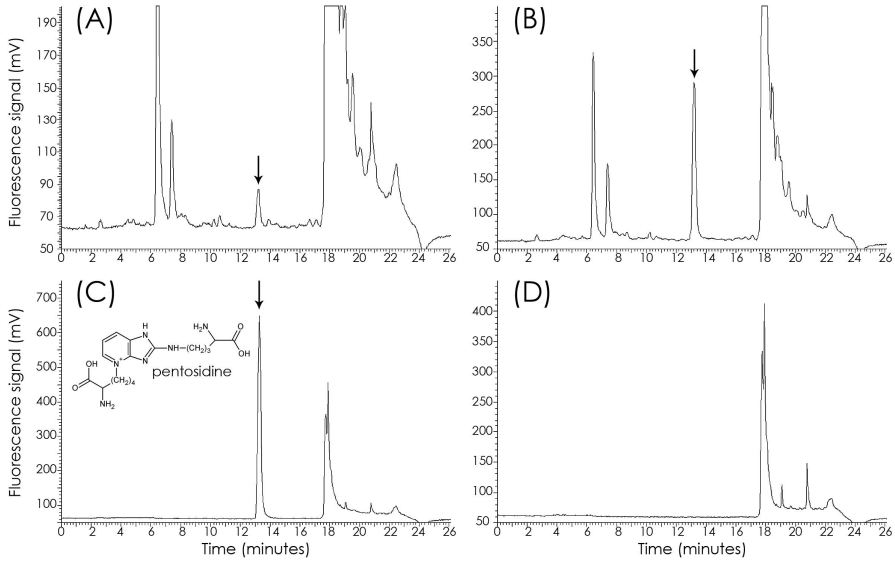

Figure 2A.1 Representative chromatograms of a standard solution of pentosidine (C), a plasma sample from a healthy control (A), a uremic patient on peritoneal dialysis (B) and a blank sample (D). The elution of pentosidine is indicated with an arrow mark with a peak area (and concentration) of pentosidine in A=314,197 (0.60 pmol/mg protein), in B=3,638,638 (6.06 pmol/mg protein), in C=8,901,011 (460 nM) and in D=0 (0 pmol). Pentosidine is clearly baseline separated which makes time consuming manual integration afterwards not necessary.

Stability of pentosidine

To make large number of measurements within one run possible, we tested the stability of pentosidine under different conditions. The stability of pentosidine was tested in solvents with neutral pH, acidic pH (pH=2.5) and basic pH (pH=10.6). Pentosidine was at least stable for 35 hours in all the tested solvents when samples were stored in the auto-injector at 6°C (figure. 2A.2).

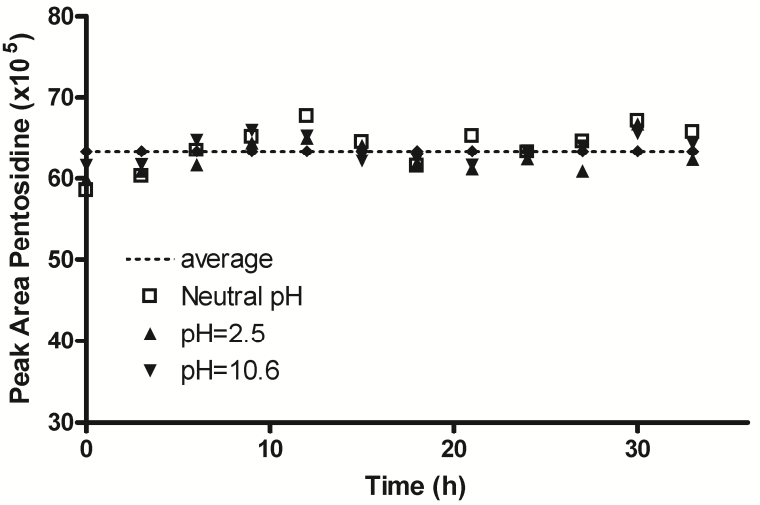

Figure 2A.2 The stability of pentosidine at different pH. Pentosidine was diluted in citric acid buffer (pH=2.5), in citrate buffer (pH=10.6) and in water to a final concentration of 32 nmol/L. Pentosidine concentration was measured upon a storage at 6°C for 35 hours.

Linearity and lower limit of quantification

Linearity of the detection of pentosidine was tested in water (with and without hydrolysis) and matrix by adding pentosidine standard during preparation of water and different plasma samples (table 2A.1 and figure 2A.3). The slope, tested in 4 different plasma samples and in water measured on two different days, was 19685±3.4% (mean±CV%). Acid hydrolysis had no effect on the peak areas of pentosidine, therefore calibration was carried out with non-hydrolysed standards. Also no matrix effect was observed as tested in different plasma samples (table 2A.1 and figure 2A.3). The limit of detection (signal-to-noise: 6) of pentosidine was 2.2 nmol/L or 0.02 pmol/mg protein, corresponding to a concentration of 0.02 pmol/mg protein.

Table 2A.1 Calibration curves of pentosidine in different matrices

Matrix	Slope	Y-intercept	r^2	Conc. Range (nmol/L)
Water (without hydrolysis)	19359	-	0.9997	0-200
Water (with hydrolysis)	18300	-	0.9991	0-200
Plasma A	19571	483386 (*24.7)	0.9988	0-200
Plasma B	20082	550906 (*27.4)	0.9984	0-200
Plasma C	19849	1027646 (*51.8)	0.9983	0-75
Plasma D	20630	473838 (*23.0)	0.9959	0-75
Mean	19731			
CV(%)	3.6			

* Endogenous pentosidine in plasma (nmol/L)

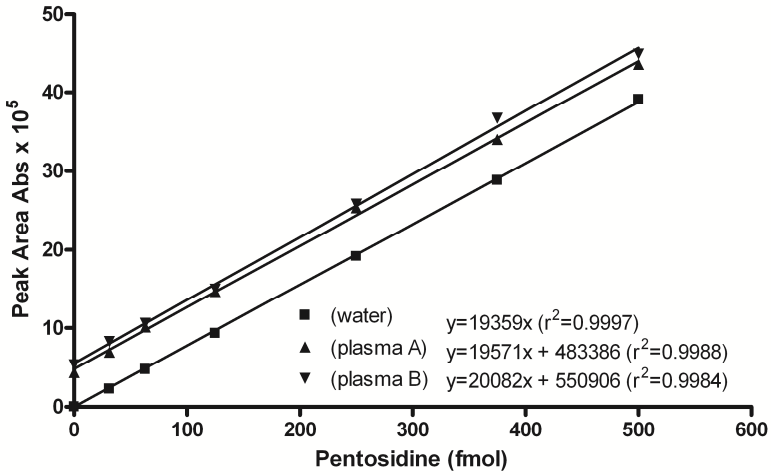

Figure 2A.3 Calibration curves of pentosidine in water and in plasma samples. Linearity of the detection of pentosidine was tested in water and in two different plasma samples by the addition of pentosidine standard (0-200 nmol/L) during sample preparation. No Matrix-effect was observed. Y-axis intercept in both plasma sample shows endogenous pentosidine (resp. 24.7 nmol/L in plasma A and 27.4 nmol/L in plasma B).

Recovery and precision

The intra-assay variation of the method was determined in two different plasma samples analysed in one batch during one day. The intra-assay variation was 6.5% as determined in a plasma sample (n=9) with a mean concentration of 0.47 pmol/mg protein and 2.0% in a plasma sample (n=10) with a mean concentration of 1.27 pmol/mg protein. The inter-assay variation of the method was determined in two different plasma samples divided into batches and analysed during different days.

The inter-assay variation was 3.1% as determined in a plasma sample (n=10) with a mean concentration of 0.43 pmol/mg protein and 1.6% in a plasma sample (n=10) with a mean concentration of 1.40 pmol/mg protein. Recovery of pentosidine was 102±10% as determined in 4 different plasma samples (table 2A.2).

Table 2A.2. Recovery of pentosidine in 4 different plasma samples.

Matrix	Added amount (fmol*)	Measured (fmol*)	Recovery (%)
Plasma A	500	563	101
	375	439	102
	250	327	108
	125	189	106
	63	131	119
	0	58	-
Plasma B	500	580	102
	375	474	108
	250	333	106
	125	193	99
	63	136	108
	0	70	-
Plasma C	188	318	100
	125	261	104
	63	190	94
	31	166	110
	0	131	-
Plasma D	188	262	104
	125	186	96
	63	123	91
	31	89	71
	0	67	-
		Mean Recovery (%)	102
		Recovery SD (%)	10

(* per injected volume of 10 µL)

Comparison of pentosidine concentration between cases and controls

We analyzed protein bound pentosidine in 24 healthy volunteers and in 24 uremic patients on peritoneal dialysis. The median (IQR) concentration of protein bound pentosidine was significantly higher in the peritoneal dialysis patients than in the healthy control group 3.05 (2.03-3.92) pmol/mg protein and 0.21 (0.19-0.33) pmol/mg protein, respectively, $p<0.00001$) (figure 2A.4).

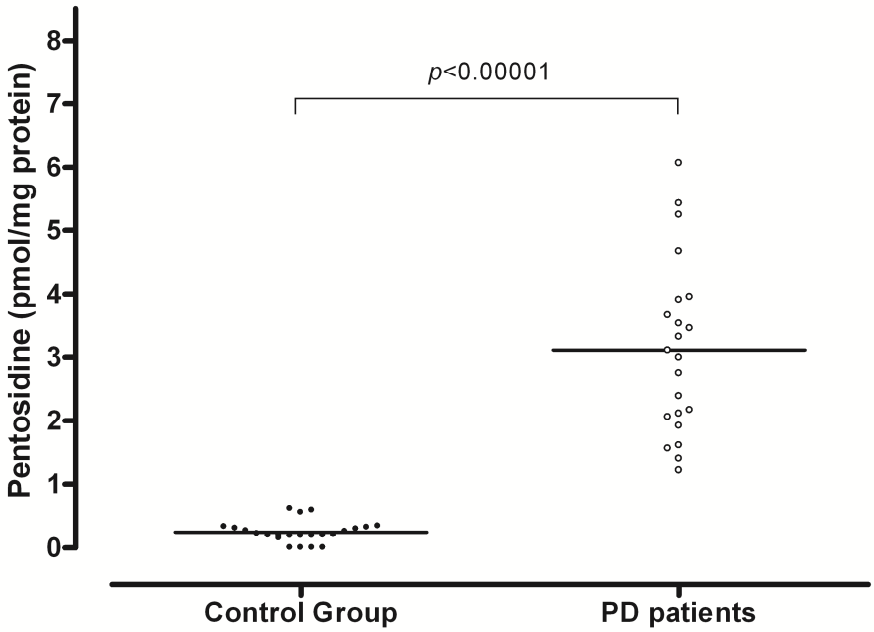

Figure 2A.4 Protein bound pentosidine concentration in plasma of healthy controls and in peritoneal dialysis (PD) patients. Data are presented as median (line) and separate datapoints.

Discussion

We describe here a rapid, simple and reliable method to determine protein bound pentosidine in plasma by a single step RP-based HPLC separation with fluorescent detection. The combination between a very simple sample preparation and a short run-time makes this method a strong and easy tool to determine protein bound pentosidine in a large number of plasma samples.
Many techniques have been used to quantify pentosidine[10-14,19,21-23,30] with some disadvantages such as long run-times[10,13,14,23], elaborate sample preparation[16] or analysis on expensive LC-MS/MS equipment[19,30].

Two main advantages of using "normal" reversed phase chromatography instead of the ion-pair based chromatography, which is commonly used in this field[9,10,12-15,23,25], is a very clean chromatogram and a very stable retention-time. Indeed, we found very clean chromatographs, with baseline separation of pentosidine in plasma, which makes afterwards time-consuming manual integration unnecessary. With a retention time of only 13.24±0.07 min and a run-to-run time of approximately 30 min, we can run, unattended, 48 samples a day.

Another advantage of the described sample preparation is that it is also suitable for the simultaneous analysis of protein-bound pentosidine with HPLC-fluorescence and for protein bound AGEs such as Nε-(carboxymethyl)lysine (CML) and Nε-(carboxyethyl)lysine (CEL) with LC-MS/MS technique[18]. Only 50 µL of plasma is needed for both methods. After hydrolysis the samples can be split; one part for the HPLC-fluorescence analysis of pentosidine and one part for the LC-MS/MS analysis.

With this method we measured protein bound pentosidine in healthy volunteers and patients on peritoneal dialysis with a limit of detection of 0.02 pmol/mg protein, which is comparable with limit of detection as described in literature[15,20,22]. The mean plasma concentration of protein bound pentosidine in healthy controls as measured with the method described above was 0.21 pmol/mg protein. The absolute concentrations of protein bound pentosidine in healthy controls described in literature are divergent, ranging from 0.95 to 2.0 pmol/mg protein[10,31-33] and our value of 0.21 pmol pentosidine per mg protein is lower. Differences in the characteristics of the controls and a lack of an international pentosidine standard may explain the differences. Therefore, direct comparison of the absolute levels of pentosidine as measured in different studies is difficult. The 15-fold increase in plasma protein bound pentosidine in uremic patients in comparison to healthy controls is in agreement with previous results[9,34,35-32].

The detection of AGEs is currently of much experimental and clinical interest, in particular because data of few epidemiological studies have demonstrated associations of plasma levels of AGEs with vascular complications[5,6].
Although studies demonstrating a causal role of AGEs in the development of cardiovascular disease are limited, it might be that specific AGEs are risk factors of cardiovascular disease. In accordance with this, high serum levels of AGEs predict increased coronary heart disease mortality in non-diabetic women[5] and a recent study demonstrated that serum pentosidine concentration is an independent prognostic factor for heart failure 6. However, AGEs in these studies were measured with immunoassays and AGEs measurements with immunoassays should be interpreted with care.
In conclusion, we describe a fast, simple and reliable method for the quantitation of pentosidine. This method may help to obtain a better understanding of the putative effects of pentosidine in the pathophysiology of different diseases such as vascular complications and to test whether pentosidine measurements can be used to identify patients with a high risk for poor outcome and may thus help in risk stratification.

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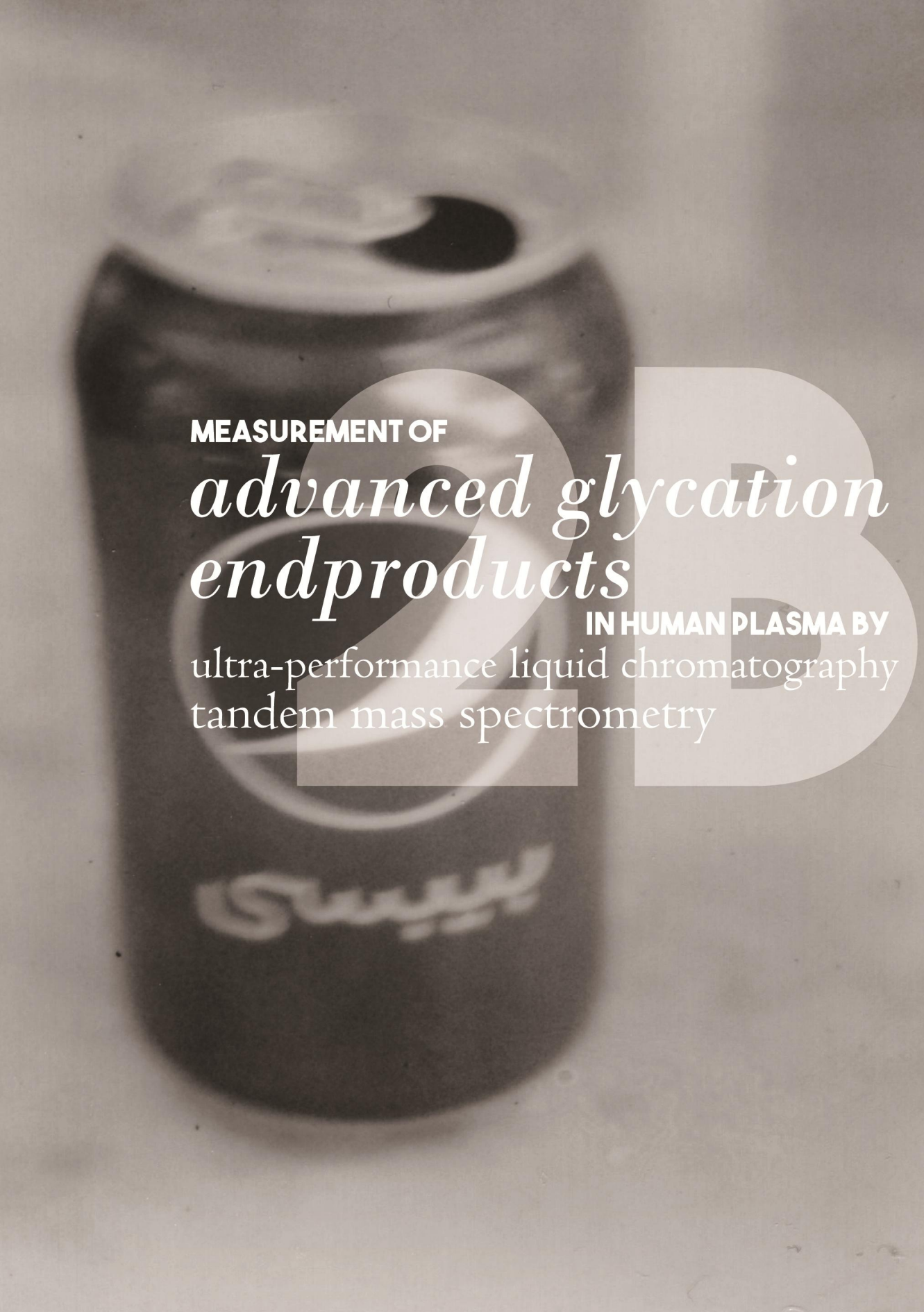

2B

MEASUREMENT OF *advanced glycation endproducts* IN HUMAN PLASMA BY ultra-performance liquid chromatography tandem mass spectrometry

2B

Measurement of advanced glycation endproducts in human plasma by ultra-performance liquid chromatography tandem mass spectrometry*

Published as supplemental data*:

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[#] Equal contribution

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Abstract

Experimental and histological data suggest a role for advanced glycation endproducts (AGEs) in diabetic complications and in the development of age-related diseases such as inflammation, atherosclerosis and neurodegenerative disorders. To investigate these diseases in more detail a highly sensitive, selective and rapid ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the simultaneous determination of N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) in plasma is described. The nonenzymatic reaction of glucose or other reducing sugars with amino groups of the side chains of arginine and lysine in proteins leads to the formation of these AGEs, including CML, CEL and MG-H1.

Protein-bound and free plasma AGEs were determined using a butanolic derivatisation in protein hydrolysate or the supernatant of deproteinized plasma, respectively. Derivatized CML, CEL and MG-H1 were separated on a reversed-phase UPLC C18 column and detected in ESI positive multiple reaction monitoring (MRM).

Calibration curves were linear throughout selected ranges. Responsefactors for CML, CEL and MG-H1 ranged between 3.1 and 13.5 CV(%). Intra- and inter-assay CVs, in protein-bound and free plasma AGEs, were between 2.8% and 23.6%. With the use of the developed method, increased protein-bound and free plasma levels of CML, CEL and MG-H1 were found in uremic patients as compared to healthy controls.

In conclusion, the described method proofed to be suitable in studying AGEs and help us to understand the putative effects of AGEs in the pathophysiology of different diseases.

Introduction

Glycation is the nonenzymatic reaction of glucose or other reducing sugars with amino groups of proteins. The amino groups of the side chains of arginine and lysine are the primary targets for this type of posttranslational modification. Over time, the initial glycation products may undergo intramolecular rearrangements and oxidation reactions and ultimately transform into stable, so-called advanced glycation endproducts (AGEs). AGEs represent a mixture of different products such as pentosidine, N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1). AGEs have different biological functions: some are protein cross-links such as pentosidine and some, such as CML, are recognition factors for specific AGE-binding receptors. The physiological consequences of AGEs in ageing and in the etiology of diabetic complications[1] and in the development of age-related diseases such as inflammation[2], atherosclerosis[3] and neurodegenerative disorders[4] has been described.

Despite progress in this field, a more comprehensive understanding of the putative effects of AGEs in the pathophysiology of these diseases is needed. In addition, the measurement of AGEs may also help to identify patients with a high risk for poor outcome and may thus help in risk stratification[5,6]. However, progress in the field is hampered by the lack of an easy method for the detection of AGEs in the laboratory. We need a rapid, simple and reliable method for the detection of AGEs.

AGEs have traditionally been detected by enzyme-linked immunosorbent assay (ELISA)[7-13]. For several reasons the use of antisera for quantitative immunoassays of protein-bound AGEs is questionable. Reproducibility and sensitivity of such an assay are not optimal, because the specificity of the antibodies is often difficult to define and, because of steric constraints, not all AGE epitopes on the protein may be available for interaction with the antibody[9,11]. Thus, AGE measurements with immunoassays should be interpreted with care. A better approach for the quantitative determination of specific AGEs in proteins is the use of a specific analytical technique. For the determination of AGE levels in both tissue and blood samples, high performance liquid chromatography (HPLC) measurements[14-17] and several mass spectrometry methods have been developed including gas chromatography mass spectrometry (GC–MS)[18,19], and liquid chromatography tandem mass spectrometry (LC–MS/MS)[13,17,20,21]. However, the simultaneous quantification of CML, CEL and MG-H1 in plasma or tissue, using a highly sensitive, selective and rapid analytical method, has not been reported yet.

In this paper we describe an ultra-performance liquid chromatography tandem mass spectrometry method for the simultaneous determination of CML, CEL and MG-H1 in plasma. This method is based on the derivatisation of CML, CEL and MG-H1 with 1-butanol in protein hydrolysate or the supernatant of deproteinized plasma.

Materials and methods

Materials

Boric acid, sodium borohydride, trifluoroacetic acid (TFA) and chloroform were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Butanol and methanol were obtained from Baker (Deventer, the Netherlands). Sodium hydroxide and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Water and acetonitrile were obtained from Biosolve BV (Valkenswaard, the Netherlands). CML (99%), [$^2H_2$]-CML (95.7%), CEL (98.6%), [$^2H_4$]-CEL, MG-H1 (92.6%) and [$^2H_3$]-MG-H1 (98.7%) were obtained from Polypeptide. All other reagents and solvents were of analytical or ULC/MS grade.

Sample preparation protein-bound AGEs

Whole blood was collected in sterile EDTA tubes. After centrifugation plasma was aspirated and stored at -80°C until analysis. Before analysis, plasma samples where thawed and mixed thoroughly. Twenty-five µL of plasma was mixed with 50 µL water in a 4 ml reaction vial. After addition of 200 µL of 100 mmol/L sodium borohydride dissolved in 200 mmol/L borate buffer (pH 9.2), the samples were incubated at room temperature for 2 hours. Samples were mixed and subsequently deproteinized with 1000 µL cold (4°C) TFA. After centrifugation (4300 g, 4°C, 20 min) the supernatant was carefully removed with a Pasteur pipette. For validation experiments 25 µL of a standard solution (six point calibration curve; 5250-0 nmol/L CML, 6250-0 nmol/L CEL and 14749-0 nmol/L MG-H1) was added. Samples were then hydrolyzed by adding 500 µL 6 N HCl and incubated for 24 hours at 110°C. After hydrolysis 40 µL hydrolysate and 20 µL internal standard (containing 1432 nmol/L [$^2H_2$]-CML, 1378 nmol/L [$^2H_4$]-CEL and 1322 nmol/L [$^2H_3$]-MG-H1) was mixed in a reaction vial. This mixture was evaporated to dryness under a stream of nitrogen gas at 70°C and subsequently derivatized in 100 µL 1-butanol:HCl (3:1, v/v) for 90 minutes at 70°C. Samples were then evaporated to dryness under nitrogen and redissolved in 200 µL water.

Sample preparation protein-bound lysine

Sample preparation for protein-bound lysine analysis was performed as described above with the exception of the derivatisation-step. After acid hydrolysis 10 µL hydrolysate was diluted with 800 µL water. Twenty µL of this mixture and 20 µL internal standard [$^{13}C_6$]-L-Lysine (40.16 µmol/L) was diluted with 500 µL 10 mmol/L ammonia.

Sample preparation free AGEs

Twenty-five µl of internal standard (containing 716 nmol/L [$^2H_2$]-CML, 689 nmol/L [$^2H_4$]-CEL and 661 nmol/L [$^2H_3$]-MG-H1) was mixed with 50 µl plasma. Samples were mixed thoroughly and subsequently deproteinized with 600 µl of a mixture of methanol and acetonitrile (1:3, by volume) and centrifuged at 14000 rpm for 20 min at room temperature. The supernatant was transferred to a reaction vial and further treated as described for the protein-bound AGEs.

UPLC tandem MS analysis AGEs

Derivatized CML, CEL and MG-H1 were analyzed by ultra performance liquid chromatography (Acquity UPLC, Waters, Milford, USA) and detected in ESI positive multiple reaction monitoring (MRM) mode using a Xevo TQ MS (Waters, Milford, USA). Derivatives were separated on a reversed-phase C18 column (Acquity UPLC BEH C18, 50 x 2.1 mm, 1.7 µm) with a linear gradient of 5 mmol/L ammonia and acetonitril at 48°C. The flow rate was 800 µL/min and the injection volume was 2 µL. Quantification of CML, CEL and MG-H1 was performed by calculating the peak area ratio of each unlabeled peak area to the corresponding internal standard peak area. The MRM transitions for CML, CEL and MG-H1 were respectively 317.1>186.1, 331.1>186.1 and 285.1>172.1. The MRM transitions for the internal standards [$^2H_2$]-CML, [$^2H_4$]-CEL, and [$^2H_3$]-MG-H1 were respectively 319.1>186.1, 335.1>190.1 and 288.1>172.1. Electrospray ionization was done at a capillary voltage of 0.5 kV a source temperature of 150°C and a desolvation temperature of 600°C. For qualitative and quantitative analysis, Masslynx software (V4.1, SCN 644, Waters) was used.

UPLC tandem MS analysis lysine

Protein bound AGEs were expressed in AGEs nmol/mmol LYS as per amount of protein. Therefore, underivatized lysine was analyzed by ultra performance liquid chromatography (Acquity UPLC, Waters, Milford, USA) and detected in ESI positive multiple reaction monitoring (MRM) mode using a Xevo TQ MS (Waters, Milford, USA). Lysine was analyzed on a reversed-phase C18 column (Acquity UPLC BEH C18, 50 x 2.1 mm, 1.7 µm) with a linear gradient of 20 mmol/L ammonium formate and acetonitril at 30°C. The flow rate was 800 µL/min and the injection volume was 2 µL. Quantification of lysine was performed by calculating the peak area ratio of the unlabeled peak area to the corresponding internal standard peak area. The MRM transitions for lysine and [$^{13}C_6$]- L-Lysine were respectively 147.2>84.2 and 153.2>89.2. Electrospray ionization was done at a capillary voltage of 0.5 kV a source temperature of 150°C and a desolvation temperature of 600°C.

Method validation AGEs

Linearity was determined by adding standard solution of CML, CEL and MG-H1 to water and eight different plasma samples. For protein-bound AGEs in plasma, a six-point calibration curve was prepared CML (0-5250 nmol/L), CEL (0-6250 nmol/L) and MG-H1 (0-14750 nmol/L). For free AGEs in plasma, a six-point calibration curve was prepared CML (0-525 nmol/L), CEL (0-625 nmol/L) and MG-H1 (0-1475 nmol/L). The peak area ratio of CML, CEL and MG-H1 multiplied by the concentration of each corresponding internal standard were plotted as a function of the concentration. For evaluation of inter- and intra-assay variation, a pooled EDTA plasma sample was analyzed on 8 different days (inter-assay) and 8 times on the same day (intra-assay). To investigate potential differences in protein-bound AGE concentration as measured in plasma and serum samples blood from 8 healthy volunteers was collected in anticoagulant (citrate and EDTA) and plain (serum) tubes.

Acid stability of AGEs

For protein-bound AGE analysis an acid hydrolysis is needed. To investigate acid stability of CML, CEL and MG-H1 a six-point calibration curve was prepared as described in the previous paragraph and subsequently hydrolyzed. This calibration curve was compared to a calibration curve without acid hydrolysis.

Method validation lysine

Linearity was determined by adding standard solution of lysine to water and 9 different plasma samples. A six-point calibration curve was prepared for lysine (0-198.2 µmol/L). The peak area ratio of lysine multiplied by the concentration of [$^{13}C_6$]-L-Lysine were plotted as a function of the concentration. For evaluation of inter- and intra-assay variation, a pooled EDTA plasma sample was analyzed on 11 different days (inter-assay) and 6 times on the same day (intra-assay). Method correlation, of the described lysine analysis, was tested in 44 protein-lysate samples as compared with a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, USA).

Results

UPLC tandem MS analysis AGEs and lysine

A representative chromatogram of free AGEs in an EDTA pool plasma, protein-bound AGEs in an EDTA pool plasma and a standard in water is shown in figure 2B.1 A, B and C. Retention times of CML, CEL and MG-H1 were 9.05, 9.70 and 3.71 minutes, respectively, and the corresponding stable isotopes were eluted at 9.03, 9.68 and 3.69, respectively. Retention time of lysine and the corresponding stable isotope was 0.17 minutes (chromatogram not shown).

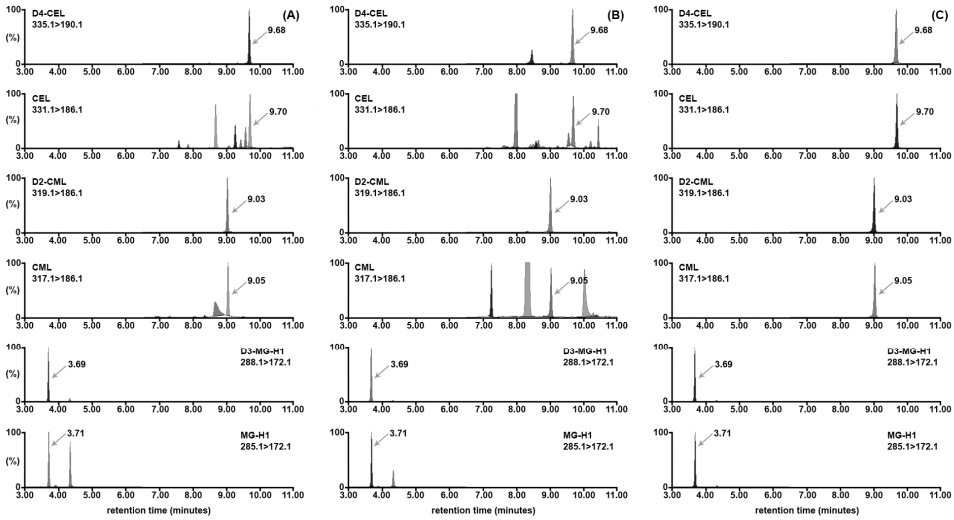

Figure 2B.1. Representative chromatograms of a standard solution of AGEs (C), an EDTA plasma sample of free AGEs (A) and an EDTA plasma sample of protein-bound AGEs (B). The elution of the AGEs are indicated with an arrow mark and corresponding retention time. Concentrations of CML, CEL and MG-H1 in the standard solution (C) were 5250, 6250 and 14750 nmol/L, in the EDTA plasma free form (A) were 65.7, 44.6 and 329.0 nmol/L and in the EDTA plasma protein-bound form (B) were 3269, 926 and 4354 nmol/L, respectively.

Method validation AGE's

The calibration curves for CML, CEL and MG-H1 were linear over the described concentration ranges (r2>0.99) in both water and plasma matrix. Mean slope (responsefactor) for protein-bound CML, CEL and MG-H1 as tested in 8 different matrices were respectively 1.064 (CV, 8.9%), 1.429 (CV, 6.0%) and 0.5718 (CV, 13.5%). Mean slope for free CML, CEL and MG-H1 as tested in 8 different matrices were respectively 1.050 (CV, 10.0%), 1.463 (CV, 3.1%) and 1.010 (CV, 3.8%).

Inter- and intra-assay variation as determined by replicate analysis of a plasma sample was given in table 2B.1.

	Intra-assay variation (n=8)				Inter-assay variation (n=8)			
	Protein-bound		Free		Protein-bound		Free	
Compound	Mean (SD) nmol/L	CV %	Mean (SD) nmol/L	CV %	Mean (SD) nmol/L	CV %	Mean (SD) nmol/L	CV %
CML	3990 (176)	4.4	66.1 (1.8)	2.8	3895 (174)	4.5	70.6 (5.0)	7.1
CEL	1278 (62)	4.8	46.9 (1.7)	3.7	1251 (182)	14.6	43.5 (2.8)	6.4
MG-H1	12072 (1005)	8.3	348.4 (13.0)	3.7	12976 (3067)	23.6	337.5 (17.1)	5.1

Table 2B.1. Intra- and inter-assay variation of protein-bound and free AGEs as determined by replicate analysis of a plasma sample.

The lower limits of quantification (s/N=10) on column for protein-bound CML, CEL and MG-H1 were 4, 4, and 7 fmol, corresponding to a concentration of 200, 200 and 340 nmol/L. The lower limits of quantification (s/N=10) on column for free CML, CEL and MG-H1 were 4, 3 and 6 fmol, corresponding to a concentration of 8.5, 4.9 and 11.8 nmol/L.

Mean protein-bound AGE concentrations, as measured in 8 different plasma and serum samples, were shown in figure 2B.2. Mean protein-bound CML concentration, as measured in citrate-, EDTA plasma and serum, was 2578, 3353 and 3026 nmol/L, respectively. Mean protein-bound CEL concentration, as measured in citrate-, EDTA plasma and serum, was 877.8, 1315 and 1008 nmol/L, respectively. Mean protein-bound MG-H1 concentration, as measured in citrate-, EDTA plasma and serum, was 12332, 13115 and 11937 nmol/L, respectively. We found higher CML and CEL concentrations, as measured in EDTA plasma compared to citrate plasma and serum. However, this was only significant for CEL ($p<0.05$). For CML we observed only a significant difference between EDTA- and citrate plasma ($p<0.05$). No statistically significant differences were found between citrate- EDTA plasma and serum MG-H1 concentrations.

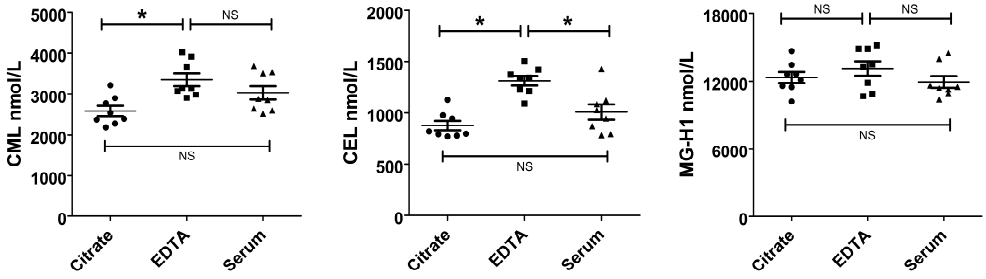

Figure 2B.2. Mean protein-bound AGE concentrations, as measured in 8 different plasma and serum samples. Data are expressed as mean ± SEM and separate datapoints (One-way ANOVA, * = $p<0.05$, NS = not significant).

Acid stability of AGEs

The calibration curves for CML, CEL and MG-H1, as tested in water after acid hydrolysis, were compared to the calibration curves without acid hydrolysis. The slope for CML, CEL and MG-H1 as tested in water, without acid hydrolysis, was 1.18, 1.59 and 1.03, respectively. For CML and CEL no differences were observed between the mean slopes of the calibration curve with or without acid hydrolysis (figure 2B.3).

For MG-H1, however, a decrease of the mean slope from 1.03 to 0.56 was observed after acid hydrolysis (figure 2B.3). Thus, under these standard conditions we found a recovery of MG-H1 of ~56%.

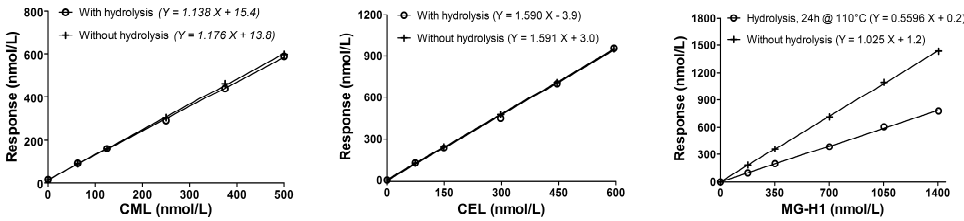

Figure 2B.3. Calibration curves in water with and without hydrolysis.

Method validation lysine

The calibration curve for lysine was linear over the described concentration range (r^2>0.99) in both water and plasma matrix. Mean slope (responsefactor) for lysine tested in 9 different matrices was 0.9936 (CV, 4.2%). Inter-assay variation as determined by replicate analysis of a plasma sample on 11 different days was 4.9 (CV%). Intra-assay variation as determined by replicate analysis (n=6) of a plasma sample on one day was 2.7 (CV%). The lower limits of quantification (s/N=10) on column for lysine was 11 fmol, corresponding to a concentration of 254 µmol/L. The described lysine analysis gave good correlation with the protein analysis as tested in 44 different protein-lysate samples (r^2=0.9304, figure 2B.4).

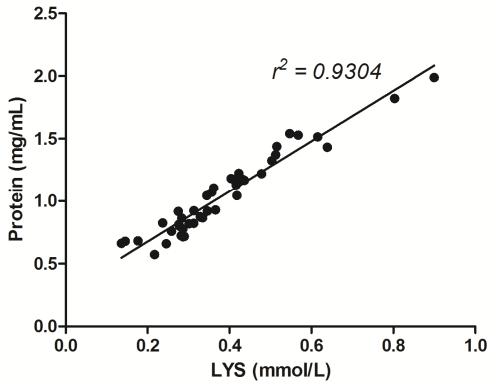

Figure 2B.4. Method correlation, as tested in 44 protein-lysate samples. Lysine concentrations correlated with protein concentrations as analyzed with a Pierce bicinchoninic acid (BCA) protein assay kit.

UPLC tandem MS analysis application

The described method has been applied to analyze human and animal plasma samples originating from a broad range of clinical trials and animal studies[22-29]. Plasma AGE concentrations were determined in patients with type 1 and type 2 diabetes[25,26,28,29], obese individuals[22] and patients with chronic obstructive pulmonary disease (COPD)[27].
As an illustration, we analyzed free and protein-bound AGEs in healthy volunteers and in patients with end stage renal disease (ESRD) receiving peritoneal dialysis (PD). As described earlier, higher plasma concentrations of protein-bound pentosidine were measured in patients with ESRD and compared to healthy volunteers[30]. Moreover, higher plasma concentrations of protein-bound and free AGEs were found in patients with ESRD compared to healthy controls[21,31]. We analyzed protein-bound AGEs in 8 healthy volunteers and in 8 patients on peritoneal dialysis. The mean concentrations of protein-bound plasma CML, CEL and MG-H1 in the PD patients were 13832, 2762 and 14105 nmol/L, respectively and were higher than in healthy volunteers 3129, 1902 and 7800 nmol/L, respectively (fig 2B.5A). This was significant for CML and MG-H1 ($p<0.0001$) but not for CEL ($p=0.1125$). We analyzed free AGEs in 9 healthy volunteers and in 9 patients on peritoneal dialysis. The mean concentrations of free plasma CML, CEL and MG-H1 in the PD patients were 1084, 1345 and 4161 nmol/L, respectively and were significantly higher ($p<0.0001$) than in healthy volunteers 68, 105 and 409 nmol/L, respectively (figure 2B.5B).

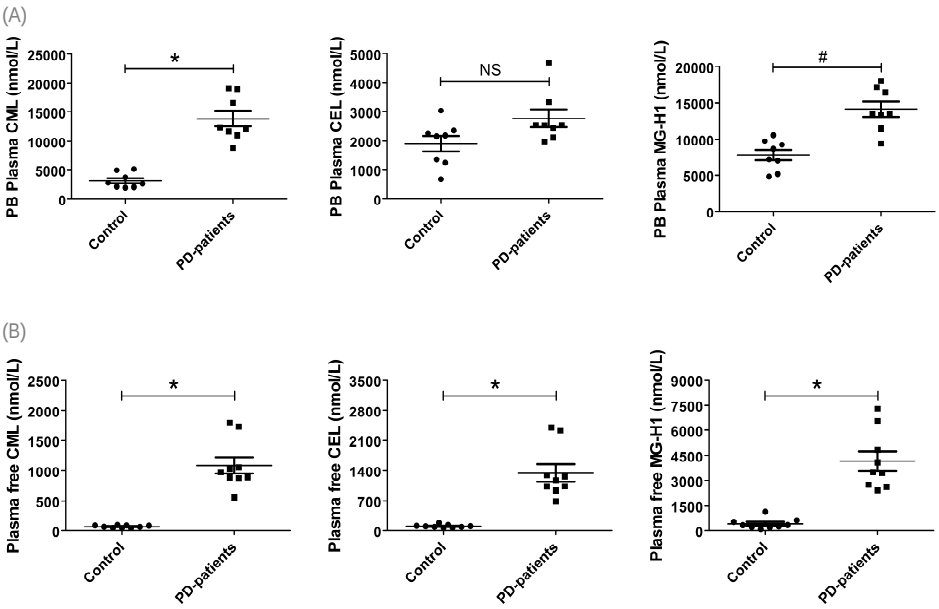

Figure 2B.5. Protein-bound (A) and free (B) CML, CEL and MG-H1 concentrations in plasma of healthy controls and in peritoneal dialysis (PD) patients. Data are expressed as mean ± SEM and separate datapoints (Unpaired t-test, *= $p<0.0001$, NS = not significant, #=$p=0.0002$).

Discussion

In 1912, Louis Camille Maillard described for the first time the reaction of protein with glucose to form advanced glycation endproducts[32]. Since his discovery a lot of research was done to investigate AGEs in the human body and their role in several diseases. To quantify AGEs in plasma and other matrices, many techniques have been described, including ELISA [7-13], HPLC[14-17], GC-MS[19,33] and LC-MS[13,17,20,21], however with some shortcomings. ELISA based techniques have several limitations quantitatively. Standardization of this method is difficult, immunoreactivity of the antibody and AGE epitope can be affected by numerous factors and sensitivity and specificity is often lacking[9]. HPLC and LC-MS uses elaborate and expensive enzymatic hydrolysis[14,17,21] which is not desirable for large cohort studies. Moreover, several analytical methods analyzed just one or two AGEs[13,20,33].
We describe here a sensitive, simple and highly selective method to determine protein-bound and free CML, CEL and MG-H1 in plasma by UPLC tandem MS.

The described UPLC tandem MS method determines simultaneously CML, CEL and MG-H1 in acid hydrolyzed plasma protein. To express these data as AGE per amount of protein, the amino acid lysine was determined in the same hydrolysate. In particular, when processing tissue samples or cell lysates it is important to correct for the amount of processed protein. Moreover, pentosidine was determined in the same hydrolysate with HPLC-fluorescence detection as described earlier[30].
To investigate the effect of anticoagulant on levels of protein-bound AGEs, citrate-, EDTA plasma and serum were considered. Protein-bound levels of CML and CEL, as measured in EDTA plasma, were higher compared to citrate plasma and serum. No differences were found between citrate-, EDTA plasma and serum levels of MG-H1. These differences are most likely due to an ion suppressing or enhancing effect on the response of these polar metabolites caused by the anticoagulant, in particular citrate and EDTA[34].
CML, CEL and MG-H1 were also determined as the free-form in the supernatant of deproteinized plasma. Twenty five µL of plasma was used for the analysis of protein-bound AGEs and 50 µL plasma was used to determine free AGEs. For each compound of interest a stable isotope internal standard was used. In this way determination is more accurate since the use of these stable isotope internal standards correct for possible coeluting polar contaminants that cause ion suppression. Moreover, a derivatisation with 1-butanol was used to gain more retention for polar compounds on reversed-phase chromatography and increases sensitivity during electro-spray ionization.
With the described method we analyzed plasma free and protein-bound AGEs in healthy volunteers and in patients with end stage renal disease (ESRD) receiving peritoneal dialysis (PD). We found increased plasma levels of both free and protein-bound AGEs in patients with ESRD as compared to healthy controls, which is in reasonable agreement with data described in literature[20,35-39]. However, absolute plasma levels differ between studies.

Differences in sample preparation, most likely protein precipitation by acid solutions compared to ultrafiltration of the plasma[21,39] and protein-bound AGEs released as free adducts via acid hydrolysis or enzymatic hydrolysis[21] can lead to different yields of AGEs. Besides, the use of standards synthetized in own laboratory facilities compared to commercially available standards can lead to absolute differences in concentrations between studies.

In conclusion, the described method proofed to be suitable in studying AGEs in several different human and animal studies[22-29] and help us to understand the putative effects of AGEs in the pathophysiology of different diseases.

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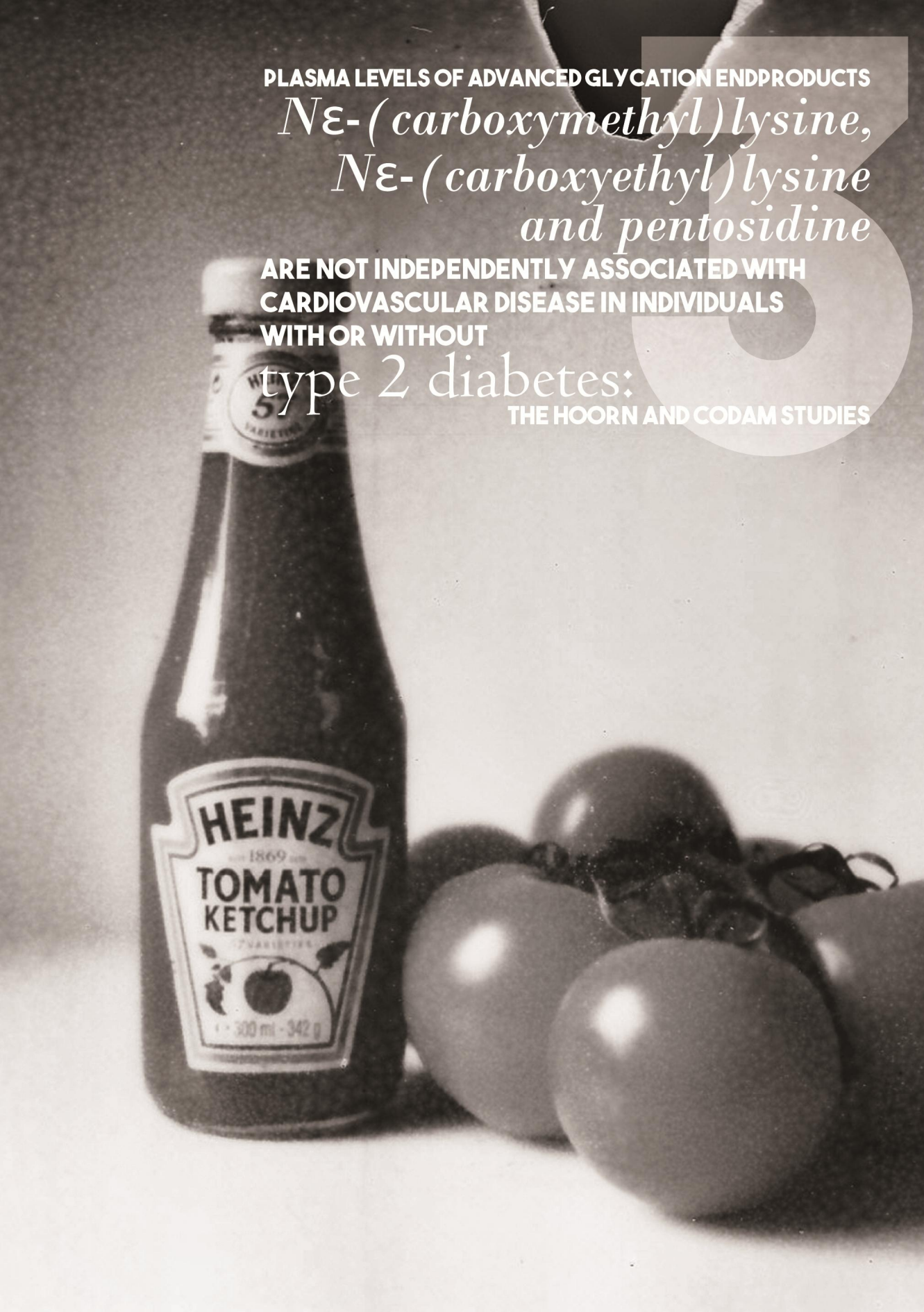

PLASMA LEVELS OF ADVANCED GLYCATION ENDPRODUCTS *Nε-(carboxymethyl)lysine, Nε-(carboxyethyl)lysine and pentosidine* ARE NOT INDEPENDENTLY ASSOCIATED WITH CARDIOVASCULAR DISEASE IN INDIVIDUALS WITH OR WITHOUT type 2 diabetes: THE HOORN AND CODAM STUDIES

3

Plasma levels of advanced glycation endproducts N^{ε}-(carboxymethyl)lysine, N^{ε}-(carboxyethyl)lysine and pentosidine are not independently associated with cardiovascular disease in individuals with or without type 2 diabetes: the Hoorn and CODAM Studies

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Abstract

Objective
Experimental and histological data suggest a role for advanced glycation endproducts (AGEs) in cardiovascular disease (CVD), particularly in type 2 diabetes (T2DM). However, the epidemiological evidence of an adverse association between AGEs and CVD remains inconclusive. We therefore investigated, in individuals with various degrees of glucose metabolism, the associations of plasma AGEs with prevalent CVD.

Research design and Methods
We measured plasma levels of protein-bound N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) and pentosidine, in participants from two Dutch cohort studies (n=1291, mean age 64.7±8.3 years, 45% women), including 573 individuals with normal glucose metabolism (NGM), 304 with impaired glucose metabolism (IGM) and 414 with T2DM. Additionally, we measured free CML, CEL and 5-hydro-5-methylimidazolone (MG-H1) in a subset of participants (n=554). Data were analyzed with multiple logistic or linear regression analyses.

Results
Protein-bound levels of CEL [32 (IQR: 25-40 vs. 28 (22-35) nmol/mmol lysine] and pentosidine [0.53 (0.43-0.67) vs. 0.48 (0.40-0.59) nmol/mmol lysine] as well as free CEL [48 (39-62) vs. 45 (36-56) nmol/L] and MG-H1 [141 (96-209) vs. 116 (84-165) nmol/L] were higher in individuals with vs. without CVD, whereas protein-bound CML was lower [33 (27-38) vs. 34 (29 39) nmol/mmol lysine]. However, these differences disappeared after adjustment for confounders. The associations did not differ consistently between individuals with- and without T2DM.

Conclusion
We found no independent adverse associations of plasma AGEs with CVD in individuals with NGM, IGM and T2DM.

Introduction

Evidence from experimental studies links advanced glycation endproducts (AGEs), a heterogeneous family of sugar-modified proteins, to cardiovascular disease (CVD) and other complications in diabetes[1]. However, epidemiological evidence of an adverse association between plasma AGEs and CVD, in individuals with or without type 2 diabetes (T2DM), remains uncertain, as studies have yielded both positive[2-7] and lack of associations[8-10]. Several factors may explain these conflicting results. Most studies were rather small, did not adjust for potential confounders, used semi-quantitative ELISA-techniques or did not measure protein-bound and free circulating forms of AGEs separately, potentially confounding results.
Therefore, we investigated, in a large combined sample of participants from two similar cohort studies: firstly, the extent to which plasma levels of the AGEs protein-bound N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) and pentosidine, measured with state-of-the-art (ultra-performance) liquid chromatography-tandem MS (UPLC-MS/MS) or HPLC[11], differed across different levels of impaired glucose metabolism [i.e. normal (NGM), impaired (IGM) and T2DM], and secondly, whether these AGEs were associated with prevalent CVD. In addition, similar analyses were conducted for free levels of CML, CEL and 5-hydro-5-methylimidazolone (MG-H1) in a subset of participants. Finally, we investigated whether these associations differed between individuals with- and without diabetes.

Research design and methods

A brief summary of the design and methods of this study is presented below, and a full presentation of the methods is available in the online supplement, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Study population

The present study included participants from the baseline examination of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) Study[12] and from the follow-up examination of the Hoorn Study[13]. These studies were designed to allow future pooling. Individuals missing data on plasma AGEs (*n*=88) or CVD status (*n*=17) were excluded from present analyses, resulting in a total of 1,291 individuals (554 from CODAM and 737 from Hoorn). Written informed consent was obtained from all participants and both cohort studies were approved by the local Medical Ethics Committees.

Measurement of plasma AGEs

Plasma AGEs were measured in EDTA plasma samples, obtained from fasting venous blood, which were stored at -80°C until analysis. We developed a new method to determine protein-bound CML and CEL and free CML, CEL and MG-H1 in plasma, using UPLC-tandem MS, which we describe in detail in Chapter 2B.

Definition of glucose metabolism status

In both cohorts participants' glucose metabolism status (GMS) was ascertained on the basis of fasting and 2-hour post-load glucose levels according to the WHO's criteria of 1999, as described in detail elsewhere[12,13]. In short, individuals with normal fasting (<6.1 mmol/L) and 2-hour post-load (<7.8 mmol/L) glucose levels were classified as having NGM. Individuals with impaired fasting glucose (6.1-7.0 mmol/L), impaired glucose tolerance (post-load glucose 7.8-11.1 mmol/L) or both were classified as having IGM. Individuals using glucose lowering medication, insulin, and/or with a fasting plasma glucose of ≥7.0 mmol/L and/or 2-hour post-load glucose of ≥11.1 mmol/L were classified as having T2DM.

Definition of cardiovascular disease

Prevalent CVD was defined as history of myocardial infarction, stroke or transient ischemic attack, non-traumatic limb amputation, coronary or peripheral artery bypass, angioplasty (self-reported), and/or signs of infarction or ischemia measured on a 12-lead ECG (Minnesota codes 1-1 to 1-3, 4-1 to 4-3, 5-1 to 5-3 or 7-1) or an ankle brachial index of <0.9 in either leg[12,13]. Taken together, of the individuals with CVD, 83% had coronary heart disease, 20% had cerebrovascular disease and 15% had peripheral arterial disease.

Covariates

Study covariates included waist circumference, smoking status, mean BP (MBP; calculated as 2*DBP+SBP)/3), total, HDL and LDL cholesterol, triglycerides, estimated (e) GFR (using the short Modification of Diet in Renal Disease equation[14]), presence of micro- or macroalbuminuria and use of BP-, lipid- and glucose-lowering medication, and were determined as described earlier[12,13].

Statistical analyses

All statistical analyses were carried out using the Predictive Analytics SoftWare, version 18.0 (SPSS IBM Corporation, Armonk, NY, USA). Skewed variables (i.e. CML, CEL, pentosidine, MG-H1, triglycerides) were $\log_e$ transformed prior to all analyses.

Data analyses

We used linear regression analyses to compare levels of AGEs between individuals with IGM or T2DM vs. NGM (reference group), first, after adjustments for age, sex, and cohort (model 1) and additionally for waist, smoking, MAP, blood lipids, eGFR, albuminuria and use of medication (model 2). We used logistic regression analyses to investigate the associations between plasma AGEs and prevalent CVD.

Results

General characteristics of the study population stratified according to prevalent CVD and GMS are shown in table 3.1.

Comparison of plasma AGE levels by GMS

Although crude plasma levels of protein-bound CML decreased and of protein-bound and free CEL increased with deteriorating GMS, (table 3.1), after adjustment for potential confounders (supplementary table S3.1), the levels of protein-bound CML and free CEL no longer differed significantly across GMS. However, but only after full adjustment for study covariates, protein-bound pentosidine was higher in individuals with T2DM vs. NGM [0.152 SD (0.007; 0.297)], an increase that was not clearly linear across deteriorating GMS (supplementary table S3.1).
Similar results were found when we investigated the linear associations of fasting glucose and HbA1c with plasma AGEs (supplementary table S3.2), or when we analysed the Hoorn and CODAM study separately (supplementary table S3.3).

Associations of plasma AGEs with CVD

Crude levels of protein-bound CEL and pentosidine and all free AGEs were significantly higher, whereas levels of protein-bound CML were significantly lower in individuals with compared to those without CVD (table 3.1).
However, after full adjustment for potential confounders (table 3.2), none of the protein-bound and free AGEs were significantly associated with prevalent CVD.
In analyses stratified according to GMS, we only found a significant positive association of protein-bound CEL with CVD in individuals with IGM [OR=1.37 (95% CI: 1.04; 1.81)], but an inverse association between this AGE and CVD in individuals with T2DM [OR=0.79 (0.63; 0.99)] (table 3.2). We found similar results when we analysed the Hoorn and CODAM cohorts separately (supplementary table S3.4).

Table 3.1 General characteristics of the study population stratified according to prior VCD or glucose metabolism status

	Prior CVD			Glucose metabolism status			
	No (*n*=733)	Yes (*n*=558)	P-value	NGM (*n*=573)	IGM (*n*=304)	T2DM (*n*=414)	$P_{-linear\ trend}$
Age (years)	63 ± 8	68 ± 8	<0.001	64 ± 8	66 ± 8	65 ± 8	0.007
Sex (% women)	45	45	0.911	46	45	45	0.786
NGM/IGM/T2DM (%)	50/23/27	37/24/39	<0.001	-	-	-	-
Prior CVD (%)	-	-	-	36	43	53	<0.001
Body mass index (kg/m^2)	27.9 ± 4.2	28.2 ± 4.4	0.253	26.8 ± 3.7	28.3 ± 4.1	29.5 ± 4.6	<0.001
Waist circumference (cm)	97 ± 12	98 ± 12	0.046	94 ± 11	99 ± 11	102 ± 12	<0.001
Fasting glucose (mmol/l)	6.1 ± 1.3	6.5 ± 1.7	<0.001	5.3 ± 0.4	6.0 ± 0.5	7.7 ± 1.8	<0.001
HbA1c (%)	5.9 ± 0.7	6.1 ± 0.8	<0.001	5.7 ± 0.4	5..9 ± 0.4	6.6 ± 1.0	<0.001
Glucose-lowering medication (%)	9	10	0.236	0	1.0	28.0	<0.001
Total cholesterol (mmol/l)	5.5 ± 1.1	5.5 ± 1.0	0.938	5.5 ± 1.0	5.6 ± 1.0	5.4 ± 1.1	0.219
HDL cholesterol (mmol/l)	1.3 ± 0.4	1.3 ± 0.4	0.603	1.4 ± 0.4	1.3 ± 0.4	1.2 ± 0.3	<0.001
LDL cholesterol (mmol/l)	3.5 ± 0.9	3.4 ± 0.9	0.271	3.5 ± 0.9	3.6 ± 0.9	3.4 ± 0.9	0.007
Triglycerides (mmol/l)	1.3 (0.9-1.9)	1.4 (1.1-2.0)	<0.001	1.2 (0.9-1.6)	1.4 (1.0-1.9)	1.6 (1.2-2.3)	<0.001
Lipid-lowering medication (%)	10	28	<0.001	14	17	23	0.001
Current smoking (%)	17	17	0.950	19	18	14	0.095
Systolic BP (mm Hg)	138 ± 18	147 ± 20	<0.001	136 ± 19	145 ± 18	148 ± 19	<0.001
Diastolic BP (mm Hg)	79 ± 9	80 ± 9	0.338	77 ± 9	81 ± 9	81 ± 9	<0.001
Mean BP (mm Hg)	99 ± 11	102 ± 12	<0.001	97 ± 11	102 ± 11	104 ± 11	<0.001
Hypertension[b] (%)	56	79	<0.001	53	71	81	<0.001
BP-lowering medication (%)	28	53	<0.001	27	40	54	<0.001
eGFR (ml/min/1.73m²)	84 ± 17	81 ± 19	<0.001	82 ± 15	82 ± 17	85 ± 21	0.079
Albuminuria[a] (%)	93/6/1	85/14/1	<0.001	93/6/1	90/9/1	84/15/1	<0.001
Protein-bound AGEs							
CML (nmol/mmol lysine)	34 (29-39)	33 (27-38)	0.004	34 (30-41)	33 (28-37)	31 (26-37)	<0.001
CEL (nmol/mmol lysine)	28 (22-35)	32 (25-40)	<0.001	28 (23-35)	31 (23-38)	31 (22-39)	0.021
Pentosidine (nmol/mmol lysine)	0.48 (0.40-0.59)	0.53 (0.43-0.67)	<0.001	0.51 (0.41-0.62)	0.48 (0.41-0.62)	0.49 (0.40-0.66)	0.086
Free AGEs[c]	n=399[c]	n=155[c]		n=287[c]	n=123[c]	n=144[c]	
CML (nmol/l)	77 (60-96)	83 (66-101)	0.070	78 (61-100)	77 (58-96)	80 (64-98)	0.955
CEL (nmol/l)	45 (36-56)	48 (39-62)	0.015	44 (36-55)	44 (36-56)	49 (39-64)	0.004
MG-H1 (nmol/l)	116 (84-165)	141 (96-209)	<0.001	127 (91-169)	115 (84-173)	121 (84-183)	0.932

Data are presented as means ± SD, medians (IQR) or percentages, as appropriate. *CVD*, cardiovascular disease; CEL, N$^{\varepsilon}$-(carboxyethyl)lysine; CML, N$^{\varepsilon}$-(carboxymethyl)lysine; *IGM*, impaired glucose metabolism; *MG-H1*, 5-hydro-5-methylimidazolone; *NGM*, normal glucose metabolism; *T2DM*, type 2 diabetes. [a] Normo-, micro- and macroalbuminuria. [b] Defined as SBP/DBP ≥140/90 mm Hg and/or use of BP-lowering medication. [c] Refers to data that are confined to the CODAM Study population only.

Table 3.2 Associations of plasma protein-bound and free AGEs with prevalent CVD in the total population and stratified according to glucose metabolism status.

		Total population (*n* =1,1291)			NGM (*n* =573)			IGM (*n* =304)			T2DM (*n* =366)		
Independent variable	Model	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI	*p*
Protein-bound													
Log_eCML	1	0.89	0.79; 1.01	0.064	0.96	0.78; 1.17	0.673	0.92	0.69; 1.23	0.568	0.81	0.67; 0.98	0.028
	2	0.97	0.85; 1.12	0.711	1.04	0.82; 1.33	0.726	1.15	0.82; 1.62	0.430	0.85	0.68; 1.06	0.142
Log_eCEL	1	1.04	0.93; 1.17	0.505	1.00	0.83; 1.22	0.973	1.37	1.07; 1.76	0.013	0.90	0.74; 1.10	0.313
	2	1.00	0.88; 1.14	0.982	1.07	0.86; 1.32	0.555	1.37	1.04; 1.81	0.025	0.79	0.63; 0.99	0.038
Log_ePentosidine	1	1.04	0.92; 1.17	0.587	1.22	0.99; 1.49	0.059	0.83	0.62; 1.12	0.218	0.98	0.82; 1.18	0.983
	2	1.07	0.93; 1.22	0.353	1.17	0.92; 1.48	0.202	1.01	0.75; 1.36	0.961	1.04	0.84; 1.28	0.740
Free[a]		Total population (n=554 [a])			NGM (*n*=287[a])			IGM (*n*=123[a])			T2DM (*n*=144[a])		
	Model	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI	*p*
Log_eCML	1	1.08	0.88; 1.32	0.467	1.21	0.90; 1.62	0.201	0.98	0.65; 1.47	0.902	0.96	0.66; 1.40	0.829
	2	0.96	0.76; 1.21	0.709	1.09	0.78; 1.53	0.603	1.00	0.79; 1.28	0.998	0.63	0.37; 1.08	0.095
Log_eCEL	1	1.12	0.92; 1.36	0.277	1.30	0.97; 1.73	0.079	0.94	0.60; 1.47	0.770	1.00	0.71; 1.42	0.996
	2	1.01	0.81; 1.27	0.915	1.12	0.86; 1.68	0.291	1.04	0.80; 1.36	0.880	0.72	0.45; 1.15	0.174
Log_eMG-H1	1	1.31	1.07; 1.60	0.009	1.37	1.00; 1.88	0.048	1.21	0.80; 1.83	0.375	1.25	0.89; 1.75	0.199
	2	1.21	0.96; 1.53	0.101	1.29	0.90; 1.86	0.175	1.24	0.97; 1.58	0.377	1.08	0.69; 1.68	0.741

OR, odds ratio: indicates the odds of prevalent CVD per SD increase in plasma protein-bound or free AGEs. Model 1: adjusted for cohort (in analyses with protein-bound AGEs only), age, sex and glucose metabolism status (in analyses in the total population only); Model 2: model 1 + waist, smoking, MAP, total-to-HDL-cholesterol ratio, Log_etriglycerides, eGFR, albuminuria (normo-, micro- or macro), BP-, lipid- and/or glucose-lowering medication. [a] Refers to data that are confined to the CODAM Study population

Discussion

This study had two main findings. First, after adjustments for potential confounders, plasma levels of protein-bound and free CML and CEL and free MG-H1 were not increased among individuals with deteriorating GMS. Only levels of protein-bound pentosidine were increased among individuals with T2DM only. Second, overall, none of the protein-bound and free AGEs were independently associated with prevalent CVD.

A general appraisal of the literature on associations between plasma AGEs and CVD shows inconsistent findings, with either positive[2-7] or null findings[8-10]. The same holds true for T2DM[7,8,10]. Differences between these studies may be attributable by varying degrees of glycaemic control, but the full range of HbA1c in our study was 4.7 to 11.3%, and we found no interaction between diabetic individuals with either good (HbA1c<7.5%) or poor (HbA1c>7.5%) glycemic control (data not shown). In addition, these differences could be due to small sample sizes and/or inconsistent or incomplete consideration of potential confounding factors in previously performed studies. Our findings in a rather large cohort describe a lack of adverse independent associations between plasma AGEs and CVD after adjustment for potential confounding factors. Our study thus illustrates the importance of these adjustments in cohort studies, because drawing inferences from unadjusted data would have led to different and misleading conclusions: e.g. that individuals with CVD had significantly lower levels of CML and higher levels of all remaining AGEs investigated herein (unadjusted data, table 3.1). Our study also extends previous ones by investigating the consistency of the associations within strata of deteriorating GMS. For instance, we found that plasma protein-bound CEL was positively associated with CVD in individuals with IGM, but inversely associated with CVD in individuals with T2DM. These contrasting associations are difficult to conciliate and most likely reflect a chance finding.

Several biological factors might explain the apparent contrasting findings between our study and the experimental work that has supported a causal role of AGEs in the development of (diabetes-related) vascular complications. First, many factors, such as dietary intake of AGEs[15], ageing[16], renal function[17] or use of lipid-lowering medication[18] may influence the concentrations of AGEs in tissues and plasma. In addition, plasma levels of the measured AGEs in this study may not adequately represent tissue AGE accumulation, as intracellular glycation is believed to be the major local source of AGEs [19] and not all AGEs may end up in the circulation.

To our knowledge, our study is one of the few large cohort studies investigating associations between several plasma AGEs and prevalent CVD. Despite the large sample size, the use of state-of-the-art methodology for assessment of a wide range of both protein-bound and free AGEs in plasma, and the adjustment for potential confounders, our study has some limitations. Firstly, the study population consists of a middle-aged Caucasian population at high risk for (or with prevalent) T2DM and CVD. Our findings may thus not apply to the general Caucasian population or to other ethnicities.

Secondly, given the cross-sectional design of our study, we cannot rule out the involvement of plasma AGEs in the development of incident CVD. Further (prospective) studies are warranted to fully address this issue. Indeed, we have shown that higher levels of protein-bound AGEs were associated with higher risk for incident CVD among individuals with type 1 diabetes[20].

In conclusion, plasma AGEs are not associated with prevalent CVD in two large cohorts of individuals with NGM, IGM and T2DM. Although experimental studies underlined the importance of the AGEs measured in this study in the pathogenesis of CVD, our study suggests that the use of plasma levels of these AGEs, as biomarkers for increased CVD risk, may be limited in a population-based setting. Therefore, alternative measurements of AGE-burden, such as methylglyoxal, AGEs in circulating cells, urine or tissue may better reflect the AGE production in tissues and need to be further investigated in large cohort studies.

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Appendix 3

Supplemental methods and tables

Supplementary methods

Study population

The present study included participants from the baseline examination of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) Study[1] and from the follow-up examination of the Hoorn Study[2], these studies were designed to allow future pooling. Briefly, the CODAM study, which started in 1999-2000, is an ongoing prospective cohort study designed to investigate the effects of obesity, glucose and lipid metabolism, lifestyle and genetic factors on CVD. In total, 574 individuals were selected from a large population-based cohort based on an elevated risk for T2DM and/or CVD[1]. The initial Hoorn study started in 1989 as a population-based cohort study investigating glucose metabolism status (GMS) and CVD risk factors among a sample of the general population of Hoorn, the Netherlands. In 2000–2001, a follow-up examination was conducted, to which the current analyses were confined, in a selected group of 822 individuals consisting of a random sample of individuals with NGM and IGM and all individuals with T2DM of the follow-up cohort and 188 additional individuals with T2DM from the Hoorn Screening study[2].

Individuals missing data on plasma AGEs (*n*=88) or CVD status (*n*=17) were excluded from present analyses, resulting in a total of 1,291 individuals (554 from CODAM and 737 from Hoorn). Excluded individuals more often had T2DM and on average somewhat higher BMI, waist circumference, BP and lower HDL-cholesterol levels (data not shown).

Written informed consent was obtained from all participants and both cohort studies were approved by the local Medical Ethics Committees.

Measurement of plasma AGEs

Plasma AGEs were measured in EDTA plasma samples, obtained from fasting venous blood, which were stored at -80°C until analysis. We developed and validated a new method to determine protein-bound CML and CEL and free CML, CEL and MG-H1 in plasma, using UPLC-tandem MS, which we describe in chapter 2B.

Protein-bound pentosidine was measured using HPLC with a fluorescent detector, as described in chapter 2A[3]. Concentrations of protein-bound CML, CEL and pentosidine were adjusted for levels of lysine and expressed as nmol/mmol lysine.

Definition of glucose metabolism status

In both cohorts participants' glucose metabolism status (GMS) was ascertained on the basis of fasting and 2-hour post-load glucose levels according to the WHO's criteria of 1999, as described in detail elsewhere[1,2]. In short, individuals with normal fasting (<6.1 mmol/L) and 2-hour post-load (<7.8 mmol/L) glucose levels were classified as having NGM. Individuals with impaired fasting glucose (6.1-7.0 mmol/L), impaired glucose tolerance (post-load glucose 7.8-11.1 mmol/L) or both were classified as having IGM. Individuals using glucose lowering medication, insulin, and/or with a fasting plasma glucose of ≥7.0 mmol/L and/or 2-hour post-load glucose of ≥11.1 mmol/L were classified as having T2DM. In total, 573 individuals had NGM, 304 had IGM and 414 had T2DM (n=303 newly identified and n=111 with known T2DM).

Definition of cardiovascular disease

Prevalent CVD was defined as history of myocardial infarction, stroke or transient ischemic attack, non-traumatic limb amputation, coronary or peripheral artery bypass, angioplasty (self-reported), and/or signs of infarction or ischemia measured on a 12-lead ECG (Minnesota codes 1-1 to 1-3, 4-1 to 4-3, 5-1 to 5-3 or 7-1) or an ankle brachial index of <0.9 in either leg[1,2]. Taken together, of the individuals with CVD, 83% had coronary heart disease, 20% had cerebrovascular disease and 15% had peripheral arterial disease.

Covariates

Study covariates included waist circumference, smoking status, mean BP (MBP; calculated as 2*DBP+SBP)/3), total, HDL and LDL cholesterol, triglycerides, estimated (e) GFR (using the short Modification of Diet in Renal Disease equation[4]), presence of micro- or macroalbuminuria and use of BP-, lipid- and glucose-lowering medication, and were determined as described earlier[1,2].

Statistical analyses

All statistical analyses were carried out using the Predictive Analytics SoftWare, version 18.0 (SPSS IBM Corporation, Armonk, NY, USA). Skewed variables (i.e. CML, CEL, pentosidine, MG-H1, triglycerides) were $\log_e$ transformed prior to all analyses.

Imputation of missing covariate data

A total of 88 individuals (7% of the total population) had missing values for one (n=82) or more (n=6) of the covariates. The percentage of missing values per variable varied from 0.1% (current smoking) to 3.5% (BP). We used multiple imputation chained equations to impute those values rather than perform complete case analyses to decrease selection bias and increase power of the analyses[5]. Results reported were those retrieved from pooled analyses on all five imputed datasets.

Data analyses

General characteristics of the combined CODAM and Hoorn study populations were compared between individuals with and without prior CVD and across levels of GMS with the use of ANOVA or χ^2-tests, for continuous or categorical data, respectively.
We used linear regression analyses to compare levels of AGEs between individuals with IGM or T2DM vs. NGM (reference group), first, after adjustments for age, sex, and cohort (model 1) and additionally for waist, smoking, MAP, blood lipids, eGFR, albuminuria and use of medication (model 2) to fully rule out potential confounding by these factors. We used logistic regression analyses to investigate the associations between plasma AGEs and prevalent CVD. These analyses were conducted in the whole study population and stratified according to individuals' GMS, and included adjustments for covariates as described in models 1 and 2 above. Results of these analyses are expressed in standardized regression coefficients.
We also tested whether associations differed between cohorts or between men and women by adding interaction terms to our models, but did not find any such consistent interactions (p-values for interaction were >0.1). Among the individuals with diabetes, the same was true for individuals with newly identified- and with known T2DM, and individuals with a low (<7.5%) or high (≥7.5%) HbA1c. Therefore, all results are shown without stratification for these factors.

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Table S3.1 Comparisons of levels of plasma protein-bound and free AGEs across levels of glucose metabolism.

		IGM vs. NGM			T2DM vs. NGM			
Dependent variable	Model	β	95% CI	*p*	β	95% CI	*p*	*p-linear trend*
Protein-bound								
Log_eCML	1	-0.299	-0.435; -0.164	<0.001	-0.455	-0.579; -0.331	<0.001	<0.001
	2	-0.090	-0.219; 0.040	0.175	-0.121	-0.262; 0.020	0.093	0.078
Log_eCEL	1	0.097	-0.042; 0.236	0.173	-0.022	-0.149; 0.105	0.735	0.829
	2	0.055	-0.088; 0.198	0.449	-0.090	-0.246; 0.066	0.258	0.352
Log_ePentosidine	1	-0.155	-0.291; -0.019	0.026	0.018	-0.106; 0.142	0.772	0.922
	2	-0.012	-0.145; 0.121	0.861	0.152	0.007; 0.297	0.040	0.060
Free[a]								
Log_eCML	1	-0.119	-0.323; 0.085	0.253	-0.083	-0.287; 0.112	0.404	0.329
	2	-0.124	-0.326; 0.077	0.228	-0.128	-0.380; 0.124	0.321	0.223
Log_eCEL	1	-0.061	-0.267; 0.145	0.561	0.215	0.018; 0.412	0.033	0.060
	2	-0.099	-0.302; 0.105	0.342	0.070	-0.183; 0.323	0.588	0.866
Log_eMG-H1	1	-0.168	-0.373; 0.037	0.108	-0.098	-0.294; 0.099	0.329	0.236
	2	-0.171	-0.380; 0.038	0.100	-0.134	-0.387; 0.119	0.301	0.165

β, standardized regression coefficient: indicates the difference in plasma protein-bound or free AGEs (in SD) between individuals with impaired glucose metabolism (IGM) or type 2 diabetes (T2DM) vs. normal glucose metabolism (NGM). Model 1: adjusted for cohort (in analyses with protein-bound AGEs only), age and sex. Model 2: model 1 + waist, smoking, MAP, total-to-HDL-cholesterol ratio, Log_etriglycerides, eGFR, albuminuria (normo-, micro-,or macro), and use of BP-, lipid- and glucose-lowering medication. [a]Refers to data that are confined to the CODAM Study population.

Table S3.2 Associations of plasma AGEs with fasting glucose and HbA1c.

		Fasting glucose			HbA1c		
	Model	β	95%-CI	*p*	β	95%-CI	*p*
Protein-bound							
Log_e CML	1	-0.118	-0.173; -0.063	<0.001	-0.084	-0.140; -0.027	0.004
	2	0.028	-0.020; 0.077	0.254	0.051	-0.004; 0.106	0.067
Log_e CEL	1	0.025	-0.030; 0.080	0.373	0.040	-0.016; 0.095	0.162
	2	0.018	-0.026; 0.062	0.419	0.042	-0.007; 0.091	0.094
Log_e Pentosidine	1	0.035	-0.021; 0.091	0.225	0.030	-0.026; 0.086	0.296
	2	0.050	0.002; 0.097	0.039	0.051	-0.001; 0.103	0.055
Free[a]							
Log_e CML	1	-0.021	-0.107; 0.064	0.627	0.031	-0.058; 0.120	0.495
	2	-0.015	-0.077; 0.048	0.642	0.039	-0.043; 0.120	0.352
Log_e CEL	1	0.129	0.046; 0.213	0.003	0.130	0.039; 0.221	0.005
	2	0.046	-0.016; 0.107	0.147	0.075	-0.011; 0.160	0.085
Log_e MG-H1	1	-0.031	-0.116; 0.055	0.482	0.016	-0.073; 0.104	0.724
	2	-0.024	-0.086; 0.037	0.441	0.018	-0.062; 0.098	0.655

β, standardized regression coefficient: indicates the increase in fasting glucose or HbA1c (in SD) per 1 SD increase in plasma protein-bound or free AGEs. Model 1: adjusted for cohort (in analyses with protein-bound AGEs only), age and sex. Model 2: model 1 + waist, smoking, MAP, total-to-HDL-cholesterol ratio, Log_etriglycerides, eGFR, albuminuria (normo-, micro-,or macro), and use of BP-, lipid- and glucose-lowering medication. [a]Refers to data that are confined to the CODAM Study population.

Table S3.3 Compasrisons of levels of plasma protein-bound AGEs across levels of glucose metabolism stratified according to cohort.

		IGM vs. NGM			T2DM vs. NGM		
Dependent variable	Model	β	95% CI	*p*	β	95% CI	*p*
CODAM							
Log_eCML	1	-0.250	-0.454; -0.046	0.016	-0.638	-0.833; -0.433	<0.001
	2	-0.085	-0.186; 0.015	0.397	-0.336	-0.462; -0.210	0.008
Log_eCEL	1	-0.087	-0.297; 0.124	0.421	-0.285	-0.486; -0.083	0.006
	2	-0.152	-0.263; -0.042	0.150	-0.389	-0.628; -0.149	0.005
Log_ePentosidine	1	-0.197	-0.404; 0.011	0.063	-0.013	-0.211; 0.185	0.898
	2	-0.068	-0.263; 0.127	0.503	0.041	-0.208; 0.291	0.745
Hoorn							
Log_eCML	1	-0.325	-0.507; -0.143	<0.001	-0.334	-0.496; -0.172	<0.001
	2	-0.080	-0.251; 0.091	0.359	-0.007	-0.182; 0.168	0.937
Log_eCEL	1	0.253	0.069; 0.436	0.007	0.201	0.038; 0.365	0.016
	2	0.223	0.033; 0.412	0.021	0.112	-0.083; 0.306	0.260
Log_ePentosidine	1	-0.121	-0.302; 0.059	0.188	0.058	-0.103; 0.219	0.481
	2	0.018	-0.161; 0.196	0.845	0.207	0.024; 0.389	0.027

β, standardized regression coefficient: indicates the difference in plasma protein-bound AGEs (in SD) between individuals with impaired glucose metabolism (IGM) or type 2 diabetes (T2DM) vs. normal glucose metabolism (NGM). Model 1: adjusted for age and sex. Model 2: model 1 + waist, smoking, MAP, total-to-HDL-cholesterol ratio, Log_etriglycerides, eGFR, albuminuria (normo-, micro-,or macro), and use of BP-, lipid- and glucose-lowering medication.

Table S3.4 Associations of plasma AGEs with prevalent CVD stratified according to cohort.

		CVD		
Independent variable	Model	OR	95% CI	*p*
CODAM				
Log_eCML	1	0.77	0.62; 0.95	0.013
	2	0.91	0.81; 1.03	0.453
Log_eCEL	1	0.99	0.82; 1.21	0.942
	2	0.95	0.85; 1.06	0.654
Log_ePentosidine	1	0.87	0.71; 1.06	0.173
	2	0.94	0.75; 1.18	0.597
Hoorn				
Log_eCML	1	0.96	0.82; 1.12	0.576
	2	1.00	0.84; 1.20	0.970
Log_eCEL	1	1.07	0.92; 1.24	0.397
	2	1.03	0.88; 1.22	0.697
Log_ePentosidine	1	1.16	0.98; 1.37	0.085
	2	1.17	0.98; 1.41	0.086

OR, odds ratio: indicates the odds of prevalent CVD per SD increase in plasma protein-bound AGEs.

Model 1: adjusted for age, sex and glucose metabolism status.

Model 2: model 1 + waist, smoking, MAP, total-to-HDL-cholesterol ratio, Log_etriglycerides, eGFR, albuminuria (normo-, micro-,or macro), and use of BP-, lipid- and glucose-lowering medication.


L(+) and D(-) Lactate
ARE INCREASED IN PLASMA AND URINE SAMPLES
OF TYPE 2 DIABETES AS MEASURED BY A
SIMULTANEOUS QUANTIFICATION OF
L(+) and D(-) Lactate
BY REVERSED PHASE LIQUID CHROMATOGRAPHY
Tandem Mass Spectrometry

4

L(+) and D(-) Lactate are increased in plasma and urine samples of type 2 diabetes as measured by a simultaneous quantification of L(+) and D(-) Lactate by Reversed Phase Liquid Chromatography Tandem Mass Spectrometry

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Abstract

Background:
Plasma and urinary levels of D-lactate have been linked to the presence of diabetes. Previously developed techniques have shown several limitations to further evaluate D-lactate as a biomarker for this condition.

Methods:
D- and L-lactate were quantified using ultra performance liquid chromatography tandem mass spectrometry with labelled internal standard. Samples were derivatized with diacetyl-L-tartaric anhydride and separated on a C18-reversed phase column. D- and L-lactate were analysed in plasma and urine of controls, patients with inflammatory bowel disease (IBD) and patients with type 2 diabetes (T2DM).

Results:
Quantitative analysis of D- and L-lactate was achieved successfully. Calibration curves were linear ($r^2>0.99$) over the physiological and pathophysiological range. Recoveries for urine and plasma were between 96% and 113%. Inter- and intra-assay variations were between 2% and 9%. The limits of detection of D-lactate and L-lactate in plasma were 0.7 µmol/L and 0.2 µmol/L respectively. The limits of detection of D-lactate and L-lactate in urine were 8.1 nmol/mmol creatinine and 4.4 nmol/mmol creatinine, respectively. Plasma and urinary levels of D- and L-lactate were increased in patients with IBD and T2DM as compared with controls.

Conclusion:
The presented method proved to be suitable for the quantification of D- and L-lactate and opens the possibility to explore the use of D-lactate as a biomarker.

Introduction

There are several conditions in which D-lactate can become increased in blood and urine in humans[1]. Recent studies demonstrated increased levels of D-lactate in diabetes and in infection, ischemia and trauma, suggesting the use of D-lactate as a biomarker. However, to further explore the use of D-lactate as such a biomarker there is a need of an improved method for analysing D- lactate.

Lactate has two optical isomers, L-lactate and D-lactate (figure 4.1b). L-lactate is the most abundant enantiomer of lactate. It is formed mainly during anaerobic glycolysis by conversion of pyruvate to L-lactate by Lactate dehydrogenase[2]. D-lactate is often considered as the non-physiological counterpart of L-lactate[1]. Under physiologic conditions the concentration of D-lactate is a 100-fold lower when compared to L-lactate[3]. The origin of D-lactate in human metabolism is thought to be derived from two major sources, namely degradation of methylglyoxal into D-lactate by the glyoxalase pathway and production by intestinal bacteria. Indeed, disturbances in these metabolic pathways are associated with increased levels of D-lactate[3-7]. Although some enzymes capable of metabolizing D-lactate have been described[8], its metabolism is very inefficient and D-lactate is mainly excreted in urine[1].

Methylglyoxal is a highly reactive compound formed in the process of glycolysis and lipid peroxidation. Methylglyoxal is increased in diabetes and is a major precursor in the formation of advanced glycation endproducts[9]. Methylglyoxal is degraded by the glyoxalase system resulting in D-lactate. D-lactate in plasma and urine has been shown to be increased in patients with diabetes[3,7]. D-lactate can be used as a reflection of methylglyoxal and is much easier to measure than the very reactive methylglyoxal.

In the colon, many commensal bacteria produce D-lactate as a result of anaerobic glycolysis. Under physiological circumstances this D-lactate is further metabolized by the commensal bacteria to acetate. Therefore, D-lactate produced in the intestinal tract does not significantly contribute to levels of D-lactate in the systemic circulation under physiological circumstances[10]. However, under pathologic conditions systemic D-lactate levels may rise due to intestinal production by bacteria. In patients with ulcerative colitis, gut ischemia and appendicitis, increased levels of D-lactate have indeed been demonstrated[4-6]. The most extreme example of impaired gut permeability and bacterial overgrowth is short bowel syndrome, which is associated with D-lactate acidosis[10].

So far, D- and L-lactate have been analysed by several different techniques ranging from chiral stationary phase liquid chromatography using UV or fluorescence detection[3,11-15], enzymatic assays[7,16-21], gas chromatography mass spectrometry (GC/MS) methods[22,23], liquid chromatography mass spectrometry (LC/MS) methods[24,25] and reversed phase liquid chromatography using fluorescence detection[26]. However, these techniques have several shortcomings such as low sensitivity[11,24,27] and large sample volume[19,21,22], complex chromatographic systems[3,12,13] and long run times[3,11,26,27]. To further explore the use of D-lactate as a biomarker there is a need of an improved method for analysing D-lactate.

In this paper we describe a highly sensitive, specific and fast ultra performance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) method for the analysis of D- and L-lactate in plasma and urine without the need of a chiral stationary phase. We achieved a significant improvement over the methods described in the literature and obtained a strong tool for the analysis of D- and L-lactate in large studies. With this method we measured plasma and urine concentrations of D- and L-lactate in controls, patients with inflammatory bowel disease (IBD) and in patients with type 2 diabetes (T2DM).

Materials and methods

Chemicals and reagents

L(+)-Lactate (98%) and Dichloromethane (≥99.9%) were obtained from Sigma-Aldrich. Ammonia solution (25%) and Acetic acid anhydrous (100%) were obtained from Merck. Formic acid (p.a.), (+)-O,O'-diacetyl-L-tartaric anhydride (≥97%) (DATAN) and lithium D-lactate (≥99%) were obtained from Fluka. Water and acetonitril (ULC-MS grade) were obtained from Biosolve. [13C¬3]-Sodium L-lactate (20%, w/w in water) was obtained from Cambridge Isotope Laboratories.

Chromatographic conditions

Samples were analysed by reversed phase LC-tandem MS using an Acquity UPLC BEH C18 analytical column (100 x 2.1 mm, 1.7 µm, Waters). Detection was carried out using a Xevo TQ tandem mass spectrometer (Waters), which was operated in negative multiple-reaction-monitoring (MRM) mode. UPLC analysis was performed using a binary gradient at a flow of 0.5 ml/min using an Acquity UPLC (Waters). Solvent A was 1.5 mM ammonium formate (pH=3.6) and solvent B was acetonitril. A linear gradient was started at 99.5% solvent A, which was changed within 3 minutes to 97% solvent A. After cleaning the column with 40% solvent B during 2 minutes the column was equilibrated for 1 minute at the initial composition. Injection volume was 2 µl and column temperature was set at 31°C. Samples were kept at 6°C. Chromatograms were acquired and processed with Masslynx V4.1 SCN 644 (Waters).

Mass spectrometry conditions

MRM transitions were optimised using direct infusion of D-lactate (500 µmol/L), [$^{13}C_3$]-L-Lactate (400 µmol/L) and L-lactate (1000 µmol/L) standard solution into the tandem MS at a flow of 20 µl/min. Optimal conditions for all parents were found at a capillary voltage of 1.5 kV and a cone voltage of 10 V. The source and desolvation temperature were 150 and 450°C respectively. The cone gas flow and desolvation gas flow were 0 and 800 l/hour respectively. To establish the most sensitive daughter ions the collision energy was set at 8 eV with a collision gas flow of 0.15 ml/min. Table 4.1 shows the optimised MRM settings.

Table 4.1 MRM settings

Component	Parent Ion (m/z)	Daughter Ion (m/z)	Collision energy (eV)	Dwell (secs)
[$^{13}C_3$]-L-Lactate	307.95	91.95	8.0	0.1
D-Lactate	304.95	88.95	8.0	0.1
L-Lactate	304.95	88.95	8.0	0.1

Plasma and Urine samples

Three groups were selected for D- and L-lactate measurements. Diabetic individuals and non-diabetic controls were sex- and age matched subsets recruited from the Cohort study of Diabetes and Atherosclerosis Maastricht (CODAM). The characteristics of these subjects have been described in detail elsewhere [28]. In short, the control group (n=52) was 55.8 ± 0.7 years old, 46% female and had a HbA1C of 5.6 ± 0.1% and a fasting plasma glucose of 5.2 ± 0.1 mmol/L. Group 2, the patients with T2DM (n=52), were 56.3 ± 0.6 years old, 39% female and had a HbA1C of 6.9 ± 0.2% and fasting plasma glucose of 8.0 ± 0.2 mmol/L. Group 3 consisted of patients with IBD in remission; 32 plasma samples (52.3 ± 8.6 years, 44% female) and 34 urine samples (54.6 ± 14.1 years, 59% female). These samples were recruited from the out-patient-clinic of the Maastricht University Medical Center.
For comparison of the proposed UPLC tandem MS method with the enzymatic method, we analysed plasma and urinary D-lactate, with both methods, in rat samples. These animals were described in details elsewhere[29].

Plasma sample preparation

To 25 µl of internal standard solution (containing 434.75 µmol/L [$^{13}C_3$]-L-Lactate) 25 µl of plasma was added. Samples were mixed thoroughly and subsequently deproteinized with 600 µl of a mixture of methanol:acetonitril (1:1, by volume) and centrifuged at room temperature during 10 minutes at 14000 rpm.

The supernatant was pipetted into a reaction vial and evaporated to dryness under a gentle stream of nitrogen at a temperature of 50°C. Fifty micro liters of freshly made DATAN (50 mg/ml dichloromethane:acetic acid (4:1, by volume)) was added. The vial was capped, vortexed and heated at 75°C for 30 minutes. After 30 minutes the vial was allowed to cool down to room temperature and the mixture was evaporated to dryness with a gentle stream of nitrogen. The derivatized residue was reconstituted with 150 µl acetonitril:water (1:2, by volume).

Urine sample preparation

Twenty-five microliters of internal standard solution (containing 434.75 µmol/L [$^{13}C_3$]-L-Lactate), 25 µl urine and 300 µl of methanol was pipetted into a reaction vial. Samples were mixed thoroughly and evaporated to dryness under a gentle stream of nitrogen at a temperature of 50°C. Fifty microliters of freshly made DATAN (50 mg/ml dichloromethane:acetic acid (4:1, by volume)) was added. The vial was capped, vortexed and heated at 75°C for 30 minutes. After 30 minutes the vial was allowed to cool down to room temperature and the mixture was evaporated to dryness with a gentle stream of nitrogen. The derivatized residue was reconstituted with 300 µl acetonitril:water (1:2, by volume).

Method validation

Linearity of the detection of D- and L-lactate was tested in water and matrix by adding D- and L-lactate standard to water and during preparation of plasma or urine samples (table 4.2). Calibration curves were obtained by linear regression of a plot of the analyte concentration (x) vs the peak-area ratio of the analyte/internal standard area (y). For both the analytes, [$^{13}C_3$]-L-lactate was used as internal standard.
The lower limit of detection was determined by calculating the concentration at a signal to noise ratio of six (s/N: 6, injection volume: 2 µl).
For recovery experiments, standard solutions of D- and L-lactate were added to urine or plasma and subsequently prepared as described in the sample preparation section.
The intra-assay variation of the method was determined in two different plasma and urine samples (n=10) analysed in one batch during one day. The inter-assay variation of the method was determined in two different plasma and urine samples divided into batches and analysed during 10 different days.
Freeze-thaw stability was tested in two different plasma and urine samples by snap-freezing these samples in liquid nitrogen and thawing them for 5 subsequent cycles.
To investigate the stability of plasma and urine samples, stored at 6°C in the autosampler, replicate injections of two different plasma and urine samples were done every hour during 24 hours.

Determination of fasting plasma glucose, Hba1C and urinary creatinine

After an overnight fast, plasma glucose concentrations (mmol/L) were measured with a hexokinase glucose-6 phosphate dehydrogenase method (ABX Diagnostics, Montpellier, France). Hba1C (%) was determined by ion-exchange high-performance liquid chromatography (HPLC) (Bio-Rad, Veenendaal, the Netherlands). Fasting plasma glucose concentrations and Hba1C were determined in the CODAM participants only.
Urinary D- and L-lactate concentrations were expressed as µmol/mmol creatinine. Creatinine concentration in urine was analysed using a Beckman LX20 analyser (Beckman Coulter) based on the Jaffé reaction method[28].

D-lactate enzymatic assay

For method comparison, an enzymatic-spectrophotometric method, based on the oxidation of D-lactate tot pyruvate by NAD+ in the presence of bacterial D-lactate hydrogenase was used[21].

Statistical Analysis

The method validation data were expressed as mean and standard deviation (SD). To investigate agreement between the enzymatic and UPLC tandem MS method we used linear regression and a Bland-Altman plot after log normalisation of the rat urine samples. Limits of agreement were defined as 2 times the SD. The patient study data were expressed as mean and standard error of the mean (SEM). To detect group differences we applied analysis of variance (ANOVA) with post-hoc Bonferroni correction. P-value <0.05 was considered statistically significant.

Results

Reversed phase chromatography

D- and L-lactate DATAN derivatives yielded a baseline separation on a reversed phase UPLC column with a retention time of 2.7 minutes for D-lactate and 2.5 minutes for L-lactate. Representative chromatograms are shown in figure 4.1.

Stability of D- and L-lactate

After 5 freeze-thaw cycles no change of D- and L-lactate levels was observed, as tested in two different plasma and urine samples (data not shown).
To make large number of measurements within one run possible, we tested the stability of D- and L-lactate when samples were stored in the auto-injector at 6°C. D- and L-lactate were at least stable for 24 hours, no degradation was observed after replicate injections of two different plasma and urine samples (data not shown).

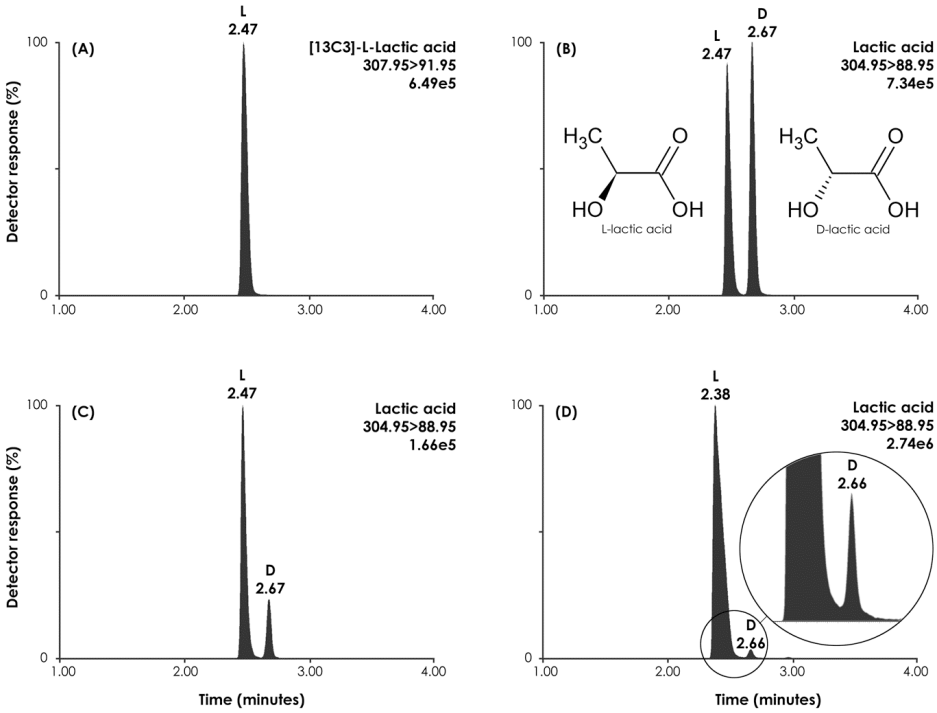

Figure 4.1. Representative chromatograms of D- and L-lactic acid derivatives [a]
(A) Internal standard [13C3]-L-lactate chromatogram (435 µmol/L, 72 pmol).
(B) Representative chromatogram of a standard solution of D- and L-lactate (351 µmol/L, 58.2 pmol and 501 µmol/L, 83.0 pmol respectively) and molecular structures of optical isomers L- and D-lactate.
(C) Representative chromatogram of a urine sample (D- and L-lactate; 14.1 µmol/L, 2.3 pmol and 74.6 µmol/L, 12.4 pmol respectively).
(D) Representative chromatogram of a plasma sample (D- and L-lactate; 11.2 µmol/L, 1.9 pmol and 1375.0 µmol/L, 227.7 pmol respectively).
[a]Injection volume: 2 µl

Linearity, lower limit of detection, recovery and precision

Linearity of the detection of D- and L-lactate was tested in matrix and water. For plasma the slope, tested in three different plasma samples and in water measured on different days, was 1.4± 6.3% (mean±CV%) for D-lactate and 0.75±3.2% for L-lactate (table 4.2). For urine the slope, tested in three different urine samples and in water measured on different days, was 1.09± 4.0% for D-lactate and 0.80±1.6% for L-lactate (table 4.2). The limits of detection of D-lactate and L-lactate in plasma were 0.65 µmol/L (108 fmol) and 0.2 µmol/L (33 fmol) respectively. The limits of detection of D-lactate and L-lactate in urine were 8.1 nmol/mmol creatinine (40 fmol) and 4.4 nmol/mmol creatinine (22 fmol) respectively. We found recoveries, for urine and plasma, between 96% and 113% (table 4.3). The validation data demonstrated inter- and intra-assay variations between 2% and 9% (table 4.4).

Table 4.2 Linearity tested in different matrices.

Matrix D-lactate	Slope[(a)]	Y-intercept	r^2	Concentration range (µmol/L)
Water	1.2927	-4.5	0.9971	0-105
Plasma A	1.4307	21.2	0.9987	0-105
Plasma B	1.3820	51.3	0.9991	0-105
Plasma C	1.5037	31.9	0.9990	0-105
Mean	1.4023			
CV(%)	6.3			
L-lactate				
Water	0.7768	-4.9	0.9996	0-3008
Plasma A	0.7566	1032	0.9998	0-6016
Plasma B	0.7164	996	0.9997	0-6016
Plasma C	0.7534	1346	0.9997	0-3008
Mean	0.7502			
CV(%)	3.2			
D-lactate				
Water	1.1265	-15.4	0.9999	0-702
Urine A	1.1092	105.24	0.9984	0-351
Urine B	1.1114	99.7	0.9999	0-702
Urine C	1.0287	69.8	0.9992	0-351
Mean	1.094			
CV(%)	4.04			
L-lactate				
Water	0.7967	0.2	0.9998	0-1002
Urine A	0.8039	60.4	0.9995	0-501
Urine B	0.7918	48.6	0.9995	0-1002
Urine C	0.7749	135.0	0.9993	0-501
Mean	0.7967			
CV(%)	1.56			

[(a)] Slope; {concentration (µmol/L)} vs. {response = (Peak Area Component/Peak Area Internal standard) * Internal standard concentration (µmol/L)}

Table 4.3 Recovery data for Plasma and Urine.

PLASMA D-lactate added[a] µmol/L (n=5)	Mean (SD) µmol/L	CV, %	Recovery, %
0	8.1 (0.2)	2.9	-
52.7	67.3 (1.2)	1.8	112.4
105.4	127.7 (3.7)	2.9	113.5
L-lactate added[a]			
0	1365 (44.6)	3.3	-
1504	2931 (26.2)	0.9	104.2
3008	4443.2 (125.1)	2.8	102.3
URINE[b] D-lactate added[a] µmol/L (n=5)	Mean (SD) µmol/mmol creatinine	CV, %	Recovery, %
0	0.91 (0.03)	3.4	-
98.8	8.03 (0.10)	1.3	110.9
197.6	14.34 (0.29)	2.0	104.7
L-lactate added[a]			
0	4.84 (0.13	2.6	-
94	10.99 (0.38)	3.5	100.6
188	16.56 (0.14)	0.8	96.0

[a] Addition of 25 µl standard solution to 25 µl plasma or urine.

[b] Creatinine concentration: 15.4 mmol/L.

Table 4.4 Precision data D- and L-lactate in plasma and urine.

Matrix Intra-assay, n=10	D-lactate Mean (SD), µmol/L	CV,%	L-lactate Mean (SD), µmol/L	CV,%
Plasma A	13.0 (0.7)	5.1	1265.3 (36.2)	2.9
Plasma B	85.7 (2.5)	2.9	6605.8 (190.4)	2.9
Inter-assay, n=10				
Plasma A	12.4 (0.6)	5.2	1338.7 (48.8)	3.6
Plasma B	85.4 (3.8)	4.4	6452.6 (245.8)	3.8
Intra-assay, n=10	D-lactate Mean (SD), µmol/mmol creatinine		L-lactate Mean (SD), µmol/mmol creatinine	CV, %
Urine A[1]	0.857 (0.03)	3.8	4.40 (0.27)	6.0
Urine B[2]	16.26 (0.48)	2.9	10.58 (0.60)	5.7
Inter-assay, n=10				
Urine A[1]	0.718 (0.04)	5.6	3.75 (0.33)	8.8
Urine B[2]	14.90 (1.05)	7.0	8.68 (0.81)	9.3

[1]Urine A, creatinine 15.4 mmol/L, [2]Urine B, creatinine 5.9 mmol/L.

Comparison of the UPLC-tandem MS method vs the enzymatic assay

We compared the proposed UPLC-tandem MS method with the enzymatic assay, by analysing plasma and D-lactate levels in rat urine with both methods. However, due to low D-lactate levels in plasma, it was not possible to analyse these samples with the enzymatic assay (data not shown). For urine, linear regression of the data resulted in the equation y = 1.08x + 50.795, with excellent correlation (r=0.985) between both techniques (figure 4.2a). However, the Bland Altman plot showed that although in the higher range both techniques are in excellent agreement, in the lower range considerately higher values were measured with the enzymatic technique as compared with the UPLC tandem MS method (figure 4.2b).

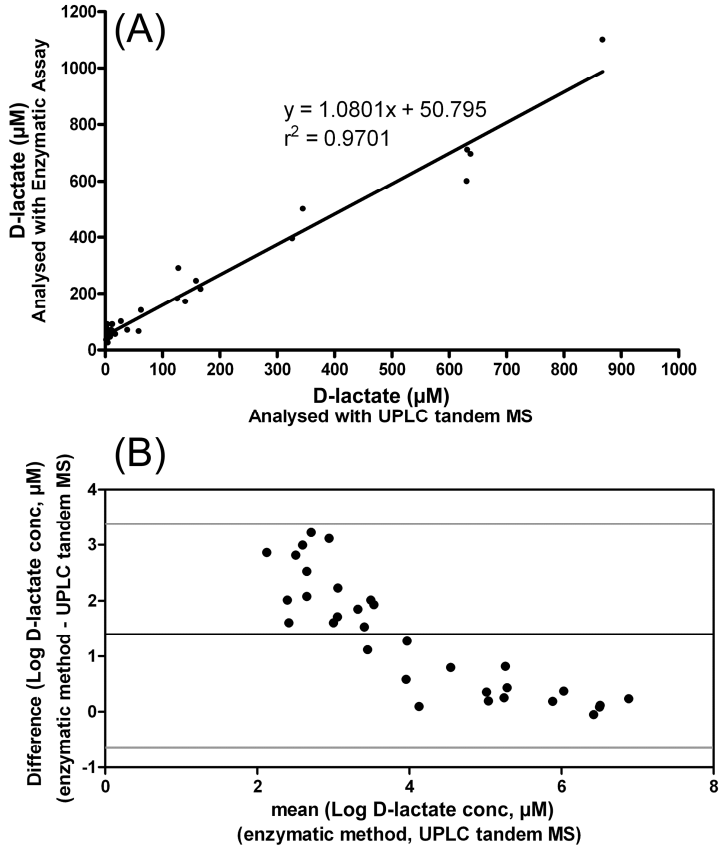

Figure 4.2. Comparison of urinary D-lactate in rat urine as measured by UPLC tandem MS and enzymatic method.
(A) Correlation between D-lactate levels measured by UPLC tandem MS and enzymatic method.
(B) Bland-Altman plot of log transformed D-lactate levels as measured by UPLC tandem MS and enzymatic method.

Comparison of urinary and plasma D- and L-lactate concentration between controls, individuals with T2DM and individuals with IBD

We next investigated D- and L-lactate plasma and urine concentration of non-diabetic controls, patients with IBD and T2DM patients (figure 4.3). In healthy controls, D- and L-lactate concentrations were 8.0 ± 0.6 and 1044.8 ± 36.7 µmol/L in plasma, respectively, and 1.1 ± 0.2 and 6.3 ± 0.9 µmol/mmol creatinine in urine, respectively (mean±SEM). In IBD patients, levels of D- and L-lactate were higher, when compared to the non-diabetic control group in both plasma (10.7±1.2 and 1172.4±74.6 µmol/L, respectively) and urine (3.1±0.8 and 11.8±1.4 µmol/mmol creatinine, respectively), which was significant for urinary L-lactate.

In T2DM patients, the concentrations of D- and L-lactate were significantly higher in plasma (12.3 ± 0.8 and 1534.7 ± 67.5 µmol/L, respectively) and urine (3.4 ± 1.0 and 12.1 ± 2.0 µmol/mmol creatinine, respectively). Both plasma and urinary D-lactate levels, as determined in T2DM and controls, correlated with HbA1C (r=0.392, p<0.001 and r=0.421, p<0.001, respectively) and fasting plasma glucose (r=0.360, p<0.001 and r=0.416 p<0.001, respectively).

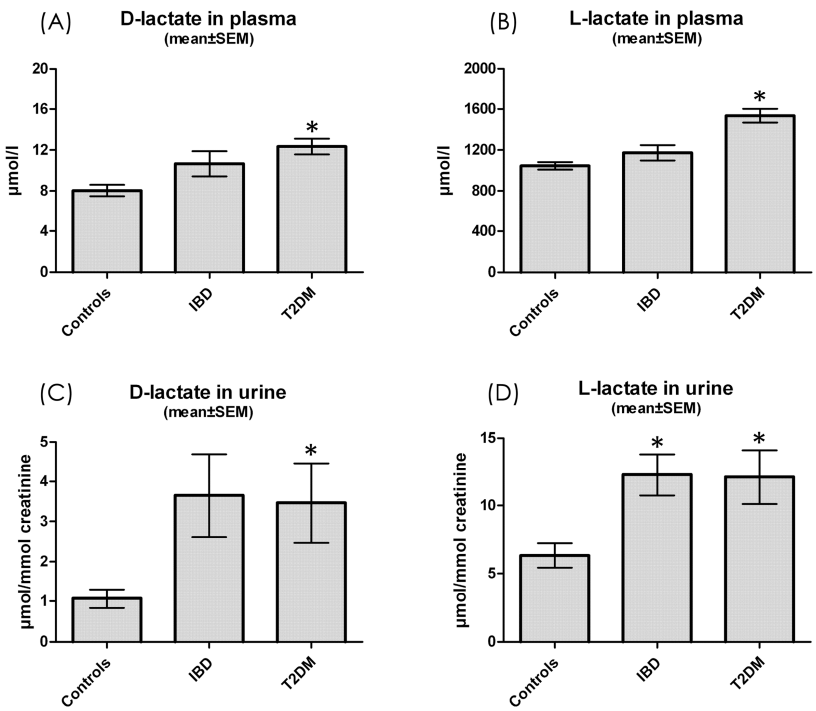

Figure 4.3. Urinary and plasma D- and L-lactate concentrations of controls, patients with inflammatory bowel disease (IBD) and patients with type 2 diabetic patients (T2DM). Data are presented as Mean±SEM, *P<0.05.

Discussion

We describe here a rapid, sensitive and highly specific method for the simultaneous determination of D- and L-lactate in plasma and urine by UPLC MS/MS. The derivatisation of D- and L-lactate with DATAN makes it possible to separate both enantiomers on a reversed phase based analytical column. This results in a robust chromatographic system without the need of a column-switching or solid phase extraction (SPE) pre-sample clean-up. We found that urinary and plasma levels of D- and L-lactate were significantly increased in T2DM as compared with non-diabetic controls. In addition, we observed higher L- and D-lactate levels in patients with IBD, but only significant for urinary L-lactate.

Many other techniques have been used to quantify D- and L-lactate, with several disadvantages, such as long run-times[3,11,26,27], large sample volume[19,21,22,24,27], a column-switching pre-separation technique[3,12,13] or low sensitivity[11,24,27]. Moreover, a disadvantage of the enzymatic method is that it is not possible to measure D- and L-lactate in a single run.

Chiral stationary phase liquid chromatography has been applied for the enantiomeric separation of D- and L-lactate[11,14,25]. SPE or pre-reversed phase liquid chromatographic separation are obligatory for good chiral chromatographic performance[3,12,13,29]. Furthermore the shorter lifetime, higher cost and difficult selection of a suitable chiral column[30,31] has made an alternative method for enantiomeric separation desirable. More recently Cevasco et al.[26] used a reversed phase liquid chromatography method for separation of D- and L-lactic acid. However; a run-to-run time of 35 minutes and an obligatory SPE sample preparation step makes this method less feasible for large cohort studies. Moreover, the used derivatisation reagent was not commercially available and had to be synthesized before use.

Anhydrides of tartaric acid were used successfully as chiral derivatisation reagents of hydroxy acids and other enantiomeric compounds[32-34]. In this paper we describe the derivatisation of the enantiomeric D- and L-lactate with DATAN. This derivatisation step results in a highly sensitive and specific D- and L- lactate derivative which is baseline separated on a UPLC reversed phase column and detected with tandem MS. The advantage of this technique, as compared to the described methods in the literature, is that there is no need for sample clean up or pre-separation of the sample matrix and only 25 µl of sample is necessary. Also the highly efficient and specific fragments of these DATAN derivatives generated in the collision cell, is an improvement against the non-derivatized analysis of D- and L-lactate with LC/MS[25]. With a run-to-run time of 6 minutes we established a fast and reliable method suitable for measuring D- and L-lactate in large cohort-studies.

The D- and L-lactate concentrations we measured in plasma and urine from healthy controls are in reasonable agreement with data obtained by other techniques[3,18,19,25]. Indeed, we found an acceptable correlation of the new UPLC tandem MS method with the enzymatic assay in urine samples.

However, the enzymatic assay is not adequately sensitive for lower levels of D-lactate, as reflected in the Bland-Altman plot. The enzymatic method measures higher levels of D-lactate than the UPLC tandem MS in the lower range.
D-lactate was not significantly increased in patients with IBD compared with non-diabetic controls in both plasma and urine. Another study, however, found a significant increase of D-lactate in hospitalised patients with active IBD 6. This difference may be explained by the fact that the patients we have included were in remission. In addition, due to the relatively small sample size, the power to detect statistically differences was low.
We found a statistically significant increase of urine and plasma levels of D- and L-lactate in T2DM as compared with non-diabetic controls. The fact that both D-lactate and L-lactate are increased in patients with T2DM suggests that the hyperglycaemic state is an important source of D-lactate elevations in diabetes. L-lactate is mainly formed during glycolysis by conversion of pyruvate to L-lactate by lactate dehydrogenase. D-lactate is an endproduct of the metabolism of methylglyoxal, formed during hyperglycaemia, by the glyoxalase pathway[35]. In line with this, we demonstrate that D-lactate correlates significantly with HbA1C, a marker for prolonged hyperglycaemia. However, based on our small study we cannot definitely conclude whether D-lactate is merely a reflection of methylglyoxal, gut-flora, or both, as several possible residual confounding factors such as BMI and gut permeability may explain the differences we observed between our patient groups.
Methylglyoxal is produced in small amount from carbohydrates, fat and protein metabolism. It is has been demonstrated that methylglyoxal is the most important precursor in the formation of advanced glycation endproducts. Methylglyoxal and methylglyoxal-derived advanced glycation endproducts are believed to be implicated in the development of diabetic vascular complications. Because D-lactate is elevated in diabetes and may be used as an indicator of methylglyoxal, the measurement of D-lactate needs to be evaluated in cohort studies with D-lactate as a possible predictor of diabetic complications. In addition, mechanistic studies are needed to elucidate the relative contribution of several metabolic pathways to the total urinary and plasma D-lactate pool, in both healthy and diabetic individuals.
In conclusion, this specific measurement of D- and L-lactate shows promise in the investigation of diabetes and metabolic diseases.

Acknowledgements

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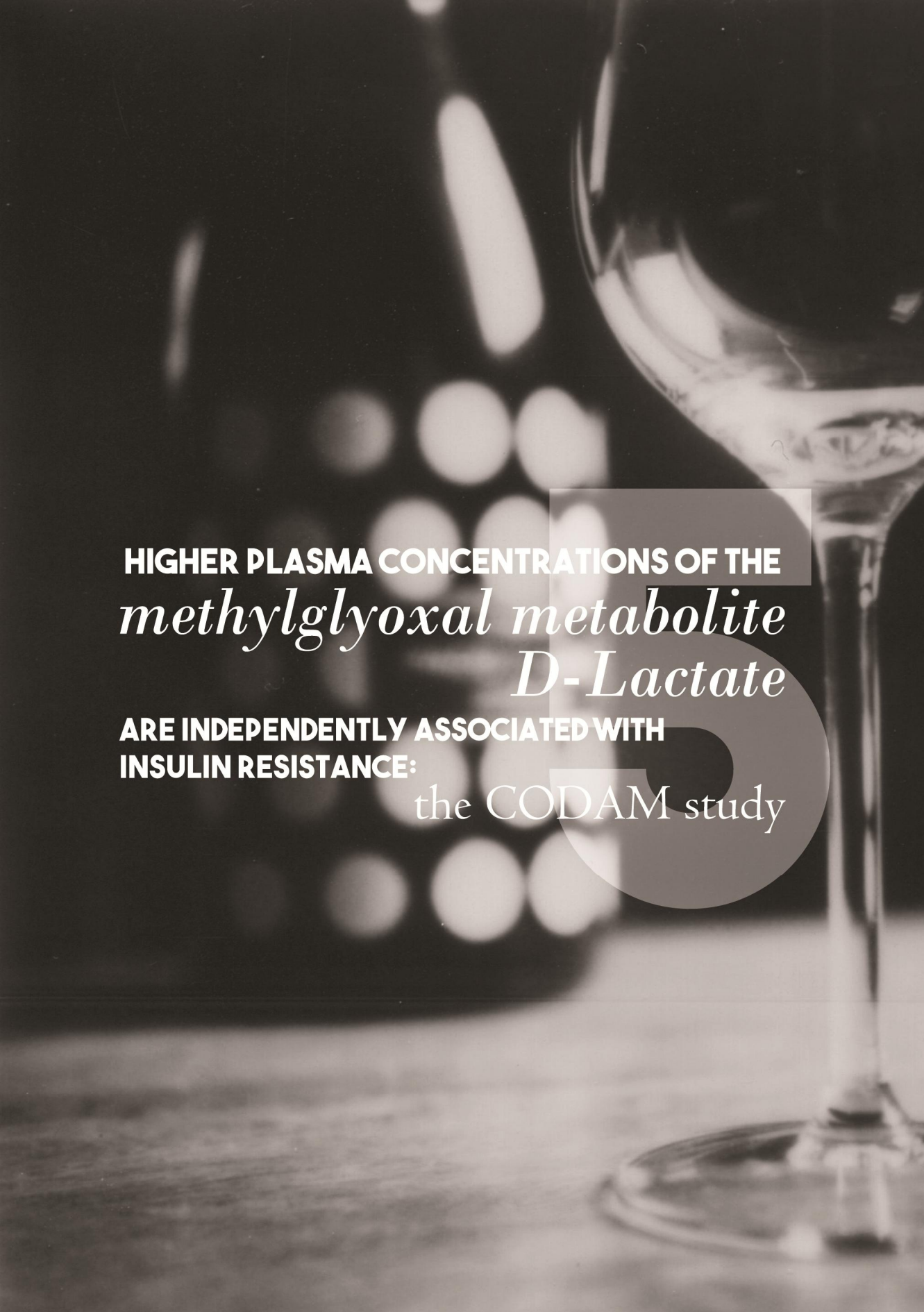

5

HIGHER PLASMA CONCENTRATIONS OF THE *methylglyoxal metabolite D-Lactate* ARE INDEPENDENTLY ASSOCIATED WITH INSULIN RESISTANCE: the CODAM study

5

Higher plasma concentrations of the methylglyoxal metabolite D-lactate are independently associated with insulin resistance: the CODAM study

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Abstract

Objective:
Methylglyoxal (MGO), a highly reactive dicarbonyl compound generated by the spontaneous degradation of glycolytic intermediates, is a major precursor for advanced glycation endproducts and can potentially disrupt cellular functions. MGO can be detoxified by the glyoxalase system into D-lactate. Although experimental studies have shown that increased levels of MGO are associated with insulin resistance, epidemiological evidence of such an association in human studies is lacking. The aim of this study was to investigate the association between plasma D-lactate, as a reflection of plasma MGO concentrations, and insulin resistance.

Methods:
Cross-sectional, complete case analyses were performed in the Cohort study on Diabetes and Atherosclerosis Maastricht (CODAM). 513 participants were included in the study population: 59.4±6.9 years of age, 63% men, and, by design, 23% impaired glucose metabolism (IGM) and 23% type 2 diabetes mellitus (T2DM). Plasma D-lactate was measured by UPLC-MS/MS. The main outcome measure was HOMA2-IR, as a measure of insulin resistance. The association between plasma D-lactate and HOMA2-IR was studied by multiple linear regression analysis.

Results:
The prevalence of T2DM increased significantly over the tertiles of plasma D-lactate concentrations. Moreover, plasma D-lactate was positively associated with HOMA2-IR, when adjusted for age and sex (β=0.429; 95% CI: 0.350-0.507; p<0.001), as well as in the fully adjusted model, additionally adjusted for glucose metabolism status, smoking status, cardiovascular disease, use of medication (glucose-, lipid-, and blood-pressure-lowering), estimated glomerular filtration rate, waist circumference, glycated hemoglobin (HbA1C) and plasma L-lactate levels (β=0.145; 95% CI: 0.051-0.239; p=0.003). Additional data indicate that other sources of D-lactate can be excluded.

Conclusion:
We found a positive association between plasma D-lactate and HOMA2-IR, independently of putative confounders. These results suggest that MGO plays a role in insulin resistance, although direct measurement of MGO is necessary to confirm this.

Introduction

Insulin resistance with respect to glucose metabolism, is a metabolic condition defined by a reduced responsiveness to the action of insulin on glucose uptake, metabolism or storage[1]. Recently, increased levels of advanced glycation endproducts (AGEs) have been found in association with insulin resistance[1-3].

AGEs are a family of unavoidable by-products of various metabolic pathways. Methylglyoxal (MGO) has received considerable attention as the most reactive and potent AGE precursor[4,5]. MGO is a dicarbonyl compound generated by the spontaneous degradation of glycolytic intermediates during glycolysis. Under physiological circumstances, MGO can be detoxified by the glyoxalase (GLO) system. This system consists of two enzymes, glyoxalase 1 (GLO-1) and glyoxalase 2 (GLO-2), which catalyze the conversion of MGO to D-lactate[6].

Although several in vitro experiments and animal studies have indicated a substantial role for MGO in the induction of insulin resistance[1,7], the association between MGO and insulin resistance in humans has not yet been studied. However, because of the high reactivity of MGO with plasma proteins, it is difficult to obtain valid and precise measurements of MGO in plasma. Since MGO is metabolized to D-lactate, we used plasma D-lactate levels as a reflection of MGO concentrations in the current analysis[8]. We previously demonstrated that we are able to measure D-lactate in a very specific way that can efficiently distinguish between D-lactate and L-lactate[9]. MGO degradation is the major route of D-lactate formation, although D-lactate can also originate from other sources such as the intestines. In previous work, however, we found that plasma levels of D-lactate were significantly increased in patients with type 2 diabetes mellitus (T2DM) compared to healthy controls, whereas patients suffering from inflammatory bowel disease (IBD) did not show increased levels of plasma D-lactate[9]. These data underline that increased concentrations of plasma D-lactate are derived from MGO and are not a reflection of D-lactate produced by the intestines.

In this study, we investigated if plasma D-lactate, as a reflection of MGO concentrations, is increased in individuals with impaired glucose metabolism (IGM) and T2DM, and whether plasma D-lactate is associated with insulin resistance in a cohort study designed to investigate glucose metabolism and cardiovascular disease.

Materials and methods

Study population

The study population consisted of participants from the Cohort study on Diabetes and Atherosclerosis Maastricht (CODAM). This study included 574 participants who were selected from the general population, as described in detail elsewhere[10].
The cohort was enriched for impaired glucose metabolism status, as defined previously[11]. Current analyses were performed on participants who were not on insulin treatment and had complete data on all variables of interest (plasma D-lactate, HOMA2-IR, age, sex, glucose metabolism status, smoking status, cardiovascular disease, use of medication (glucose-, lipid-, and blood-pressure-lowering), estimated glomerular filtration rate (eGFR), waist circumference, glycated hemoglobin (HbA1C) and plasma L-lactate levels). Hence, the present study reports on 513 persons. The study protocol was approved by the local Medical Ethical Committee of Maastricht University Medical Centre and written informed consent was obtained from all participants.

Measurement of plasma D-lactate

Plasma D-lactate levels were measured in EDTA plasma samples from overnight fasting venous blood. Samples were analyzed by reversed phase ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), as described in detail previously[9]. The intra- and inter-assay variations were 4 and 8%, respectively.

Measurement of insulin resistance

Insulin resistance was estimated using the Homeostasis Model of Assessment – Insulin Resistance (HOMA2-IR) calculator from www.dtu.ox.ac.uk, as described previously[12].

Measurements of covariates

Plasma L-lactate levels were measured in EDTA plasma samples from overnight fasting venous blood, using UPLC-MS/MS[9]. It was measured synchronously with plasma D-lactate. Smoking behavior (never, ever or current smoker) was assessed by a questionnaire. Prior CVD and waist circumference were assessed as previously described in detail[13].

Creatinine levels were measured in EDTA plasma using a Jaffe diagnostic test (Roche Diagnostics) and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was used to calculate the eGFR[14]. Finally, HbA1C was measured using ion-exchange HPLC[10].

Use of medication

Subjects were asked to report if they were currently using medication, and, if so, to list all the medication used. All medicines were reviewed with the use of the Dutch Pharmacological Compass 2006 (www.fk.cvz.nl) and allocated into 36 categories. Medication containing the additive polyethylene glycol (PEG) was selected using the database of College ter Beoordeling van Geneesmiddelen (www.dbg-med.nl).

Definition of intestinal disease

Diseases of study participants were self-reported by questionnaire. Intestinal disease was reported when the subject suffered from any serious intestinal disease for 3 months or longer in the past 12 months.

Statistical analyses

The general characteristics of the study population were compared across tertiles of plasma D-lactate concentrations. One-way analysis of variance (ANOVA) was used for continuous variables and chi-square tests for discrete variables. Skewed variables (plasma D-lactate, HOMA2-IR, fasting plasma glucose, plasma L-lactate and triglycerides) were compared across the plasma D-lactate tertiles by Kruskal–Wallis one-way analysis of variance and were $\log^e$ transformed prior to regression analyses. Multiple linear regression analysis was used to investigate the cross-sectional association between plasma D-lactate and HOMA2-IR and results are presented as standardized regression coefficients (β). The analysis was initially adjusted for the covariates age (years) and sex (model 1), additionally adjusted for glucose metabolism status (IGM and T2DM as dummy variables), smoking status (ex-smoker and current smoker as dummy variables), prior cardiovascular disease, use of medication (glucose-, lipid-, and blood-pressure-lowering) and eGFR (model 2), further adjusted for HbA1C (model 3), and finally fully adjusted for plasma L-lactate levels (model 4). A P-value of < 0.05 was considered statistically significant. All statistical analyses were performed with IBM SPSS Statistics Software, version 20 (IBM Corporation, Armonk, New York).

Results

General characteristics of the study population stratified according to tertiles of plasma D-lactate concentrations are shown in table 5.1. The prevalence of IGM did not differ between the tertiles of plasma D-lactate levels (table 5.1). However, the prevalence of T2DM increased significantly with higher plasma D-lactate levels (p<0.001) (table 5.1). Similar results were found for fasting plasma glucose levels, HOMA2-IR, and HbA1C (all p<0.001) (table 5.1).

Table 5.1. General characteristics of the study population stratified according to tertiles of plasma D-lactate.

	Plasma D-lactate tertiles			
	Lowest (n=171)	Middle (n=171)	Highest (n=171)	P-value
Age, years	59.2 ± 6.9	59.9 ± 7.1	59.1 ± 6.8	0.43
Sex, % men	57.3	66.1	64.9	0.19
Body mass index, kg/m^2	27.3 ± 3.7	28.0 ± 4.1	30.0 ± 4.4	<0.001
Waist circumference, cm	95.5 ± 10.9	98.6 ± 11.2	103.5± 12.0	<0.001
IGM, %	23.4	20.5	25.1	0.58
T2DM, %	11.7	21.6	35.1	<0.001
Fasting plasma glucose, mmol/L	5.4 [5.0-5.8]	5.6 [5.2-6.1]	5.9 [5.4-7.1]	<0.001
HOMA2-IR	0.9 [0.7-1.3]	1.2 [0.9-1.6]	1.4 [1.0-2.3]	<0.001
Plasma D-lactate, µmol/L	5.5 [4.6-6.3]	8.5 [7.8-9.7]	14.5 [12.6-17.1]	<0.001
Plasma L-lactate, µmol/L	922 [795-1054]	1143[974-1315]	1634 [1358-1884]	<0.001
HbA_{1C}, %	5.7 ± 0.7	5.9 ± 0.7	6.1 ± 0.8	<0.001
Glucose-lowering medication, %	5.3	9.4	17.0	0.002
Prior CVD, %	21.6	26.9	32.7	0.07
Total cholesterol, mmol/L	4.2 ± 1.3	4.8 ± 1.6	5.2 ± 1.7	<0.001
Triglycerides, mmol/L	1.1 [0.8-1.5]	1.4 [1.0-1.9]	1.8 [1.4-2.4]	<0.001
Lipid-lowering medication, %	15.8	17.0	20.5	0.50
Current smoker, %	20.5	19.3	22.8	0.72
Former smoker, %	52.0	51.5	51.5	0.99
BP-lowering medication, %	28.7	38.0	46.2	0.004
eGFR, mL/min/1.73 m^2	90.5 ± 11.5	90.2 ± 12.6	93.8 ± 12.1	0.009

Data are presented as means ± SD, medians (interquartile range), or percentages, as appropriate; the ranges of the plasma D-lactate tertiles were [2.6-7.1], [7.1-10.7] and [10.7-32.4] µmol/L respectively. IGM: impaired glucose metabolism; T2DM: type 2 diabetes mellitus; HOMA2-IR: homeostasis model assessment insulin resistance; HbA1C: glycated hemoglobin; CVD: cardiovascular disease; BP: blood-pressure; eGFR: estimated glomerular filtration rate.

Plasma D-lactate concentrations were positively associated with HOMA2-IR, in the model adjusted for age and sex (Model 1: β=0.429; 95% CI: 0.350-0.507; p<0.001), as well as in a model adjusted for the additional covariates glucose metabolism status, smoking status, cardiovascular disease, use of medication, eGFR and waist circumference (Model 2: β=0.214; 95% CI: 0.147-0.280; p<0.001).

After additional adjustment for HbA1C, which had a negligible effect on the regression coefficient, plasma D-lactate remained positively associated with HOMA2-IR (Model 3: β=0.210; 95% CI: 0.143-0.276; p<0.001).
Addition of plasma L-lactate levels to model 3 resulted in a relatively large decrease in the regression coefficient (Model 4: β=0.145; 95% CI: 0.051-0.239; p=0.003), although the association between plasma D-lactate and HOMA2-IR remained significant (Table 5.2).

Table 5.2. Association between plasma D-lactate (µmol/L) and HOMA2-IR.

Dependent variable	Model	β	95% CI	P-value
HOMA2-IR	1	0.429	0.350-0.507	<0.001
	2	0.214	0.147-0.280	<0.001
	3	0.210	0.143-0.276	<0.001
	4	0.145	0.051-0.239	0.003

The standardized regression coefficient β represents one standard deviation (SD) increase in HOMA2-IR per SD increase in plasma D-lactate.
Model 1: adjusted for age and sex;
Model 2: model 1 + glucose metabolism status, smoking status, cardiovascular disease, use of medication (glucose-, lipid-, and blood-pressure-lowering), estimated glomerular filtration rate and waist circumference;
Model 3: model 2 + HbA1C;
Model 4: model 3 + plasma L-lactate levels.

Although MGO degradation is the major route of D-lactate formation, D-lactate can also be produced by other pathways. We already found in our previous study that patients suffering from IBD did not show increased levels of plasma D-lactate. However, we additionally analyzed our study population with regard to intestinal disease. There were no differences in prevalence of intestinal disease between the plasma D-lactate tertiles (T1: 1.8%, T2: 4.7%, T3: 2.9%; p=0.29), nor was there a difference in the proportion of intestinal diseased participants treated for this disease (T1: 50%, T2: 57%, T3: 44%; p=0.88).
In addition to the intestinal route, D-lactate can also be formed from PEG, which is used as an additive for several medications. To exclude PEG as a source of D-lactate production, we selected all medication in our cohort which was enriched with PEG, and analyzed whether there were more users of PEG-contaminated medication in the higher tertile of plasma D-lactate. We found that the users of PEG-contaminated medication were equally distributed between the tertiles of D-lactate (T1: 35%, T2: 40%, T3: 46%; p=0.14). Since PEG is often added to BP-lowering medication, we performed the same analyses for this specific group of medication. Again, no difference in amount of users was found between the D-lactate tertiles (T1: 57%, T2: 66%, T3: 60%; p=0.58).

Discussion

In this cohort study we demonstrated that plasma D-lactate levels are increased in patients with T2DM. Additionally, we found a positive association between plasma D-lactate levels and HOMA2-IR, independently of putative confounders.

This is the first study that has examined the association between MGO and insulin resistance in a large human cohort study, using plasma D-lactate as a reflection of MGO concentrations. We observed a significantly higher prevalence of T2DM across the tertiles of plasma D-lactate concentrations. A similar trend was observed for fasting plasma glucose levels, HOMA2-IR, and HbA1C, supporting the hypothesis that persons with abnormal glucose metabolism have high plasma D-lactate levels. These positive associations are in line with our previous study, in which we demonstrated that T2DM patients have increased plasma and urine levels of D-lactate[9] and with studies showing that the concentration of D-lactate typically increased in cells cultured in high glucose concentrations[5,8].

We found a strong association between plasma D-lactate and HOMA2-IR, which was independent of confounders. Although adjustment for plasma L-lactate levels resulted in a relatively large decrease in the regression coefficient, the association between plasma D-lactate and HOMA2-IR remained significant, demonstrating an association between plasma D-lactate probably derived from MGO metabolism and insulin resistance. Our results are in agreement with several experimental studies showing that the major AGE-precursor MGO potentially contributes to the development of insulin resistance. It has been demonstrated that administration of MGO to rats caused impaired insulin signaling and induced insulin resistance[15-18]. In addition, chronic infusion of MGO caused β-cell dysfunction[18]. Next to the in vivo studies, in vitro experiments with 3T3 adipocytes and INS-1E pancreatic cells showed that IRS-1 phosphorylation and PI3K activity were dose-dependently reduced by treatment with MGO, indicating its contribution to the pathogenesis of insulin resistance[17,19].

Our finding that plasma D-lactate is positively and independently associated with insulin resistance is in line with findings from recent studies, which described a positive association between AGEs and insulin resistance[1-3]. However, we cannot establish whether these associations are causal due to the cross-sectional design of the study. Further follow-up data on these or other patient groups would, of course, be invaluable, in testing whether our findings are probably due to causal relationships between MGO and insulin resistance. However, this cross-sectional study may serve as a reasonable starting point to further explore these associations. Plasma D-lactate may be, as a reflection of plasma MGO, an early marker of insulin resistance.

The main limitation of our study is that we were not able so far to measure MGO directly in these plasma samples. However, it is very likely that an increase in D-lactate concentrations is indicative of increased flux of MGO formation, as was demonstrated in erythrocytes[8]. Furthermore, our additional analyses showed that we can exclude other sources than MGO for the production of D-lactate.

Previous work and current results indicate that both the intestines as well as PEG-contaminated medication do not contribute to plasma D-lactate levels.
In summary, our data show for the first time that plasma D-lactate levels are associated with HOMA2-IR, independently of putative confounders. Together with findings from previous studies, our results suggest that higher levels of MGO play an important role in the etiology of insulin resistance, although this has to be confirmed in future research.

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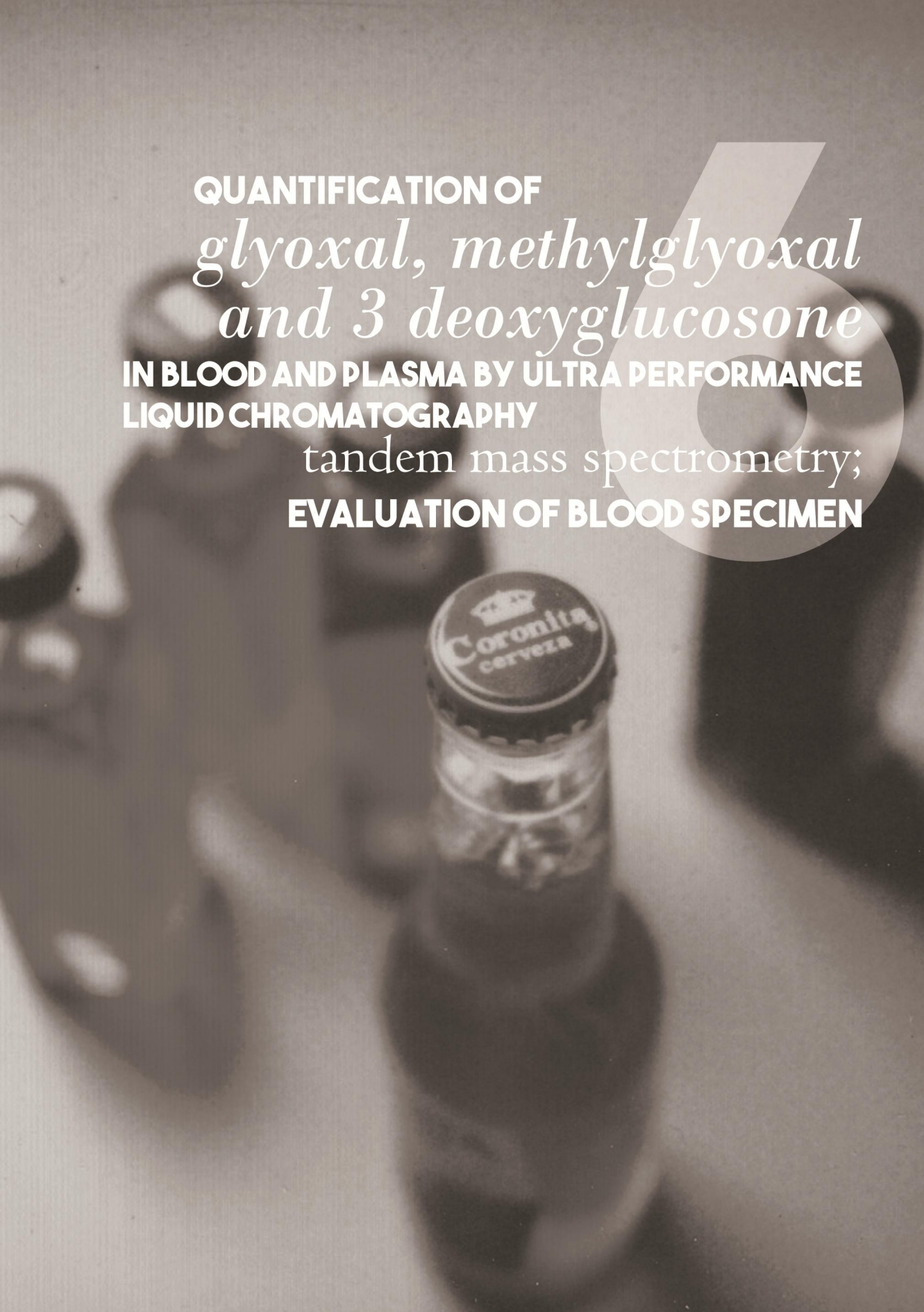

6

QUANTIFICATION OF *glyoxal, methylglyoxal and 3 deoxyglucosone* IN BLOOD AND PLASMA BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY tandem mass spectrometry; EVALUATION OF BLOOD SPECIMEN

6

Quantification of glyoxal, methylglyoxal and 3-deoxyglucosone in blood and plasma by ultra-performance liquid chromatography tandem mass spectrometry; *evaluation of blood specimen*

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Abstract

Background: The reactive α-oxoaldehydes glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) have been linked to diabetic complications and other age-related diseases. Numerous techniques have been described for the quantification of α-oxoaldehydes in blood or plasma, albeit with several shortcomings such as the need of large sample volume, elaborate extraction steps or long run-times during analysis. Therefore, we developed and evaluated an improved method including sample preparation, for the quantification of these α-oxoaldehydes in blood and plasma with ultra-performance liquid chromatography tandem mass spectrometry (UPLC MS/MS).

Methods: EDTA plasma and whole blood samples were deproteinized using perchloric acid (PCA) and subsequently derivatized with o-pheneylenediamine (oPD). GO, MGO and 3-DG concentrations were determined using stable isotope dilution UPLC MS/MS with a run-to-run time of 8 minutes. Stability of α-oxoaldehyde concentrations in plasma and whole blood during storage was tested. The concentration of GO, MGO and 3-DG was measured in EDTA plasma of non-diabetic controls and patients with type 2 diabetes (T2DM).

Results and conclusion: Calibration curves of GO, MGO and 3-DG were linear throughout selected ranges. Recoveries of these α-oxoaldehydes were between 95 and 104%. Intra- and inter-assay CVs were between 2 and 14%.
To obtain stable and reliable α-oxoaldehyde concentrations, immediate centrifugation of blood after blood sampling is essential and the use of EDTA as anticoagulant is preferable. Moreover, immediate precipitation of plasma protein with PCA stabilized α-oxoaldehyde concentrations for at least 120 minutes. With the use of the developed method, we found increased plasma concentrations of GO, MGO and 3-DG in T2DM as compared to non-diabetic controls.

Introduction

Prolonged exposure to hyperglycemia has detrimental effects on various cellular functions and is believed to be the most important factor in the development of vascular complications in diabetes. One of the hypotheses about how hyperglycemia leads to complications is the formation of advanced glycation endproducts (AGEs). In addition to the formation of AGEs by the classical Maillard reaction, α-oxoaldehydes such as methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucocone (3-DG) are also known to form AGEs[1-3]. These α-oxoaldehydes and AGEs have been linked to diabetic complications as well as other age-related human diseases[4-10]. To further investigate the putative role of these α-oxoaldehydes in several diseases a specific, rapid and sensitive method is needed.

So far, α-oxoaldehydes have been analyzed by several techniques including reversed phase liquid chromatography with fluorescence or UV detection[2,11-15] or liquid chromatography with mass spectrometry detection[1,8,16-18]. However, these techniques have several shortcomings such as large sample volume[1,8,11,13], elaborate liquid-liquid-extraction (LLE) or solid phase extraction (SPE) steps[1,2,8,11,16,17] and long run-times[2,12,15].

In the present study we developed a highly sensitive, specific and rapid ultra-performance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) method for the analysis of GO, MGO and 3-DG. We achieved a significant improvement over the methods described in literature with the use of a stable isotope labeled internal standard. Since variations in the blood draw and pretreatment of blood or plasma can also substantially influence the free concentrations of these α-oxoaldehydes, we evaluated blood specimen and the effect of immediate precipitation of proteins in plasma or whole blood with perchloric acid (PCA) on the concentration of α-oxoaldehydes. With an optimized method, we quantified the concentration of GO, MGO and 3-DG in EDTA plasma samples of non-diabetic controls and patients with type 2 diabetes (T2DM).

Materials and methods

Chemicals

Glyoxal (GO) solution (~40%), methylglyoxal (MGO) solution (~40%), formic acid (p.a. for mass spectroscopy) and perchloric acid (PCA, 70%, p.a.) were obtained from Sigma-Aldrich (Steinheim, Germany). D8-O-phenylenediamine (oPD) (98.6%) was obtained from CDN-isotopes (Quebec, Canada). 3-Deoxyglucosone (3-DG) (95%) was obtained from Santa Cruz (Santa Cruz, California). O-phenylenediamine was obtained from Merck (Darmstadt, Germany). Water and acetonitrile (ULC/MS quality) were obtained from Biosolve Chimie (Dieuze, France). EDTA and EDTA + sodium fluoride (NaF) tubes were obtained by Greiner Bio-One (Vacuette Premium Tubes, Kremsmünster, Austria).

Preparation internal standard oxoaldehydes

Internal standards for GO, MGO and 3-DG were not commercially available. Therefore, a derivatized internal standard was prepared by adding 140 µl GO (17.5 mmol/L), 40 µL MGO (6.5 mmol/L) and 50 µL 3-DG (5.3 mmol/L) to 50 ml 265 mmol/L formic acid. D_8-oPD was added to a final concentration of 4.3 mmol/L. This mixture was allowed to stand for 1 week at 4°C shielded from light. Aliquots of this solution were stored at -80°C until usage.

Evaluation of blood specimen

To examine the concentration of GO, MGO and 3-DG in blood and plasma samples during handling and storage, blood specimens of 2 healthy controls were collected in EDTA and EDTA + NaF tubes. Immediately upon withdrawal, blood was divided into two portions; one portion was centrifuged immediately after collection (10 minutes, 4°C, 3500 rpm) and the supernatant was stored as plasma and a second portion was stored as whole blood. At 0, 30, 60, 90 and 120 minutes, samples were analyzed for the concentration of GO, MGO and 3-DG in plasma and whole blood (figure 6.2 and 6.3). Additionally, plasma was prepared from whole blood which was stored for 0, 30, 60, 90 and 120 minutes at room temperature, and analyzed for the concentration of GO, MGO and 3-DG in plasma (figure 6.4). Furthermore, the effect of storage on the stability of GO, MGO and 3-DG concentrations was investigated, in plasma and whole blood specimens, with or without immediate precipitation of proteins with PCA.

Sample preparation

Whole blood was collected in sterile EDTA or EDTA + NaF tubes and prepared and/or stored as described in the different experiments. Before analysis, plasma samples where thawed (if needed) and mixed thoroughly. Twenty-five µl plasma was mixed with 75 µL oPD (10 mg oPD in 10 mL 1.6 mol/L perchloric acid) in an eppendorf cup. After an overnight (20 h) reaction at room temperature and shielded from light, 10 µl of internal standard solution was added. Samples were mixed and subsequently centrifuged for 20 minutes at 14000 rpm at a temperature of 4°C. Ten µl was injected for UPLC/MSMS analysis.

UPLC tandem MS

Derivatized GO, MGO and 3-DG were analyzed by ultra-performance liquid chromatography (Acquity UPLC, Waters, Milford, USA) and detected in ESI positive multiple reaction monitoring (MRM) mode using a Xevo TQ MS (Waters, Milford, USA). Derivatives were separated on a reversed-phase C18 column (Acquity UPLC BEH C18, 50 x 2.1 mm, 1.7 µm) with a binary gradient of 5 mmol/L formic acid (solvent A) and acetonitril (solvent B) at a flow rate of 800 µl/min (split-ratio: waste/tandem MS=1/1). A linear gradient was started at 99% solvent A, wich was changed within 4 minutes to 75% solvent A. After cleaning the column with 50% solvent B during 2 minutes, the column was equilibrated for 2 minutes at the initial conditions. The injection volume was 10 µL and column temperature was set at 30°C. Quantification of GO, MGO and 3-DG was performed by calculating the peak area ratio of each unlabeled peak area to the corresponding internal standard peak area. The MRM transitions for GO, MGO and 3-DG were respectively 131.1>77.1, 145.1>77.1 and 235.1>171.1. The MRM transitions for the internal standards [$^2H_4$]-GO, [$^2H_4$]-MGO, and [$^2H_4$]-3DG were respectively 135.1>81.1, 149.1>81.1 and 239.1>175.1. Electrospray ionization was done at a capillary voltage of 0.5 kV a source temperature of 150°C and a desolvation temperature of 600°C. For qualitative and quantitative analysis Masslynx software (V4.1, SCN 644, Waters, Milford, USA) was used.

Method validation

Linearity was determined by adding standard solution of GO, MGO and 3-DG to water and to 6 different plasma samples. A six-point calibration curve was prepared for GO (1458-0 nmol/L), MGO (1083-0 nmol/L) and 3-DG (2320-0 nmol/L). The peak area ratio of GO, MGO and 3-DG multiplied by the concentration of each corresponding internal standard were plotted as a function of the concentration. For the evaluation of the inter- and intra-assay variation a pooled EDTA plasma sample was analyzed on 10 different days (inter-assay) and 10 times on the same day (intra-assay). Recovery (%) was tested by adding standard solution of GO, MGO and 3-DG at two different concentration levels (729 and 182 for GO, 542 and 135 for MGO, 1475 and 369 for 3-DG nmol/L) to 6 different plasma samples. Stability of GO, MGO and 3-DG derivatives was tested by replicate injections, every 4 hours, in a pooled EDTA plasma sample. Plasma concentrations were corrected for possible formation of GO, MGO and 3-DG during storage in the autoinjector.

Subjects

Diabetic individuals and non-diabetic controls were randomly selected from the Cohort study of Diabetes and Atherosclerosis Maastricht (CODAM). GO, MGO and 3-DG measurements were performed in EDTA plasma. The characteristics of these subjects have been described in detail elsewhere 19. In short, the control group (n=10; 9 female) were non-diabetic controls of 58.4 ± 10.0 years old and the second group were patients with type 2 diabetes (T2DM) (n=10; 2 female) and were 65.3 ± 6.0 years old. After an overnight fast, plasma glucose concentrations (mmol/L) were measured with a hexokinase glucose-6 phosphate dehydrogenase method (ABX Diagnostics, Montpellier, France). Plasma glucose concentrations were 4.5 ± 0.1 mmol/L in non-diabetic controls and 10.2 ± 0.3 mmol/L in patients with T2DM.

Statistical Analysis

The method validation data and patient study data were expressed as mean ± standard error of the mean (SEM). To detect group differences we applied the Mann-Whitney U t-test. P-value <0.05 was considered statistically significant.

Results

UPLC tandem MS analysis

A representative chromatogram of a standard in water and an EDTA pool plasma sample is shown in figure 6.1A and 6.1B. Retention times of GO, MGO and 3-DG were 2.09, 2.63 and 1.67 minutes, respectively and the corresponding stable isotopes (Figure 6.1C) eluted at 2.06, 2.60 and 1.65 minutes, respectively.

Method validation

Calibration curves for GO, MGO and 3-DG were linear over the concentration ranges (r2>0.99), as described in the method section, in both water and plasma matrix. Mean slope (response factor, Rf) for GO, MGO and 3-DG tested in 6 different plasma samples were 1.036 (CV, 7.5%), 0.850 (CV, 8.2%) and 0.800 (CV, 5.6%), respectively. Mean Rf for GO, MGO and 3-DG tested in water (analyzed on 3 different days) were 1.175 (CV, 1.5%), 0.897 (CV, 2.0%) and 1.011 (CV, 0.9%), respectively.
Inter-assay variation as determined by replicate analysis of a pooled EDTA plasma sample on 10 different days was for GO, MGO and 3-DG 14.3%, 7.3% and 12.0%, respectively. Intra-assay variation as determined by replicate analysis (n=10) of a pooled EDTA plasma

sample on one day was for GO, MGO and 3-DG 4.3%, 2.9% and 2.4%, respectively. The lower limits of quantification (s/N=6) on column for GO, MGO and 3-DG were 200, 17, and 10 fmol, corresponding to a concentration of 100, 9 and 5 nmol/L, respectively.
Mean recovery, as tested in 6 different plasma samples at two different concentration levels, was shown in table 6.1.

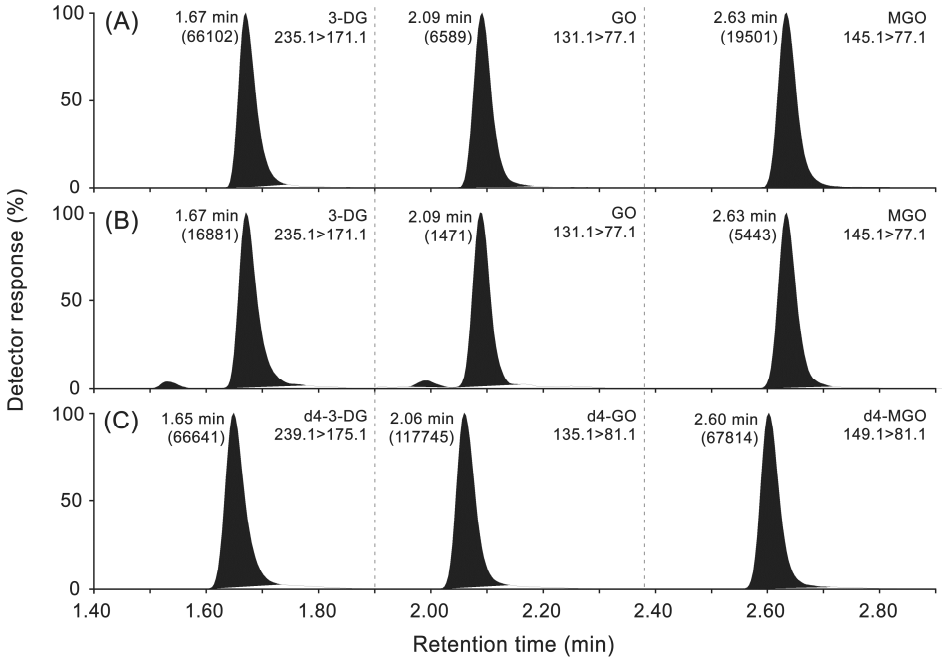

Figure 6.1 Representative chromatogram of a standard (A) of GO, MGO and 3-DG, an EDTA pooled plasma sample (B) and an internal standard (C) of [$^2H_4$]-GO, [$^2H_4$]-MGO and [$^2H_4$]-3DG.

Table 6.1 Recovery of GO, MGO and 3DG as tested in 6 different (A to F) plasma samples. Recovery was tested by adding GO, MGO and 3-DG standard, at two different concentration levels, to 6 different plasma samples.

	Added (nmol/L)	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	Mean	SD
GO high	729	102.6	97.2	99.6	108.8	96.6	102.3	101.2	4.5
GO low	182	101.8	104.3	99.3	132.2	101.2	83.5	103.7	15.8
MGO high	541	103.5	89.8	98.8	101.8	104.0	98.9	99.5	5.2
MGO low	135	108.7	80.1	95.9	99.7	108.3	77.5	95.0	13.5
3-DG high	1475	110.4	104.5	101.9	110.2	80.7	81.8	98.3	13.6
3-DG low	369	126.3	113.4	99.5	119.5	86.3	72.4	102.9	20.7

Evaluation of blood specimen

Stability of GO, MGO and 3-DG in whole blood

In figure 6.2 the stability of GO, MGO and 3-DG in whole blood is shown. When proteins in blood, collected in EDTA or EDTA + NaF tubes, were directly precipitated with PCA, the concentration of GO, MGO and 3-DG were stable for at least 120 minutes. Without immediate precipitation, the concentration of GO and MGO in blood increased over time in EDTA + NaF tubes and MGO in blood was also increased in EDTA tubes. A slightly decrease of 3-DG in time was observed in both EDTA + NaF and EDTA tubes.

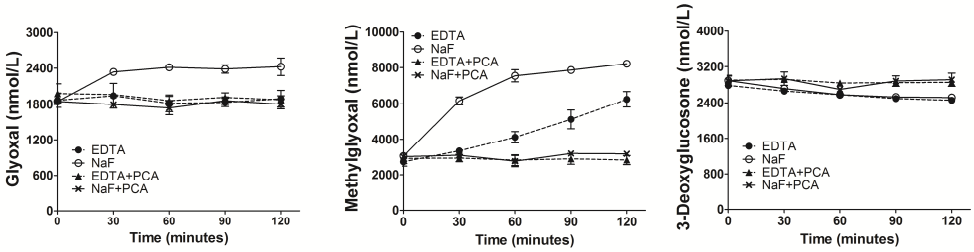

Figure 6.2 Stability of GO, MGO and 3-DG in whole blood (EDTA or EDTA + NaF) after immediate precipitation with PCA or whole blood processed after standing at room temperature for the indicated time followed by precipitation. Data presented as mean ± SEM.

Stability of GO, MGO and 3-DG in plasma (stored as whole blood)

In figure 6.3 the stability of GO, MGO and 3-DG in plasma stored as whole blood is shown. The concentration of GO was at least stable for 120 minutes in EDTA + NaF tubes, without the need for immediate centrifugation and precipitation. In contrast, the concentration of GO decreased over time in EDTA tubes. MGO was at least stable for 120 minutes in EDTA tubes, without the need for immediate centrifugation and precipitation with PCA, but increased over time in EDTA+NaF tubes. In both EDTA+NaF and EDTA tubes a decrease of 3-DG was observed.

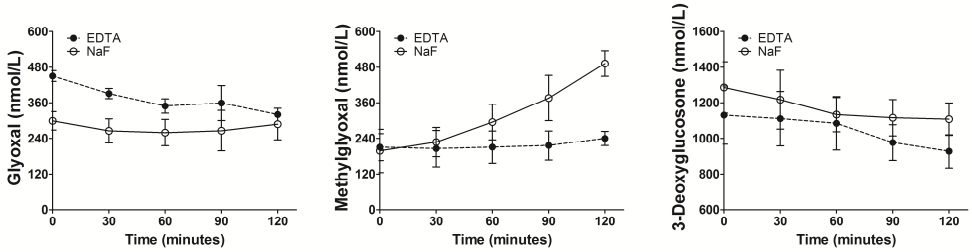

Figure 6.3 Stability of GO, MGO and 3-DG in plasma (stored as whole blood at room temperature, EDTA vs EDTA + NaF). Data presented as mean ± SEM.

Stability of GO, MGO and 3-DG in plasma

To study the stability of GO, MGO and 3-DG in plasma, blood specimen were immediately centrifuged after withdrawal. When the protein fraction in plasma was immediately precipitated with PCA, the three oxoaldehydes were stable for at least 120 minutes in EDTA plasma (figure 6.4). Without immediate precipitation with PCA, MGO remained stable for 120 minutes, but a decrease was observed for GO and 3-DG after 60 minutes.

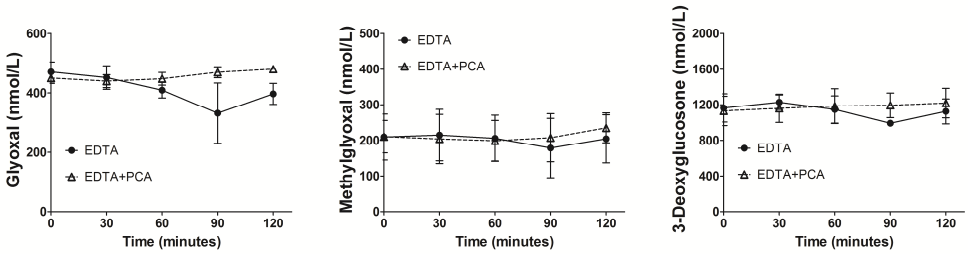

Figure 6.4 Stability of GO, MGO and 3-DG in plasma (EDTA) after immediate precipitation with PCA or without immediate precipitation. Data presented as mean ± SEM.

Concentration of GO, MGO and 3-DG in non-diabetic controls vs patients with type 2 diabetes

When compared to healthy controls, EDTA plasma from patients with type 2 diabetes showed significantly increased concentrations of MGO (277 ± 9 vs 212 ± 8 nmol/L) and 3-DG (2217 ± 81 vs 1046 ± 37 nmol/L) (figure 6.5). Plasma GO concentration was also increased in T2DM vs non-diabetic controls (514 ± 49 vs 406 ± 26 nmol/L), although not significant.

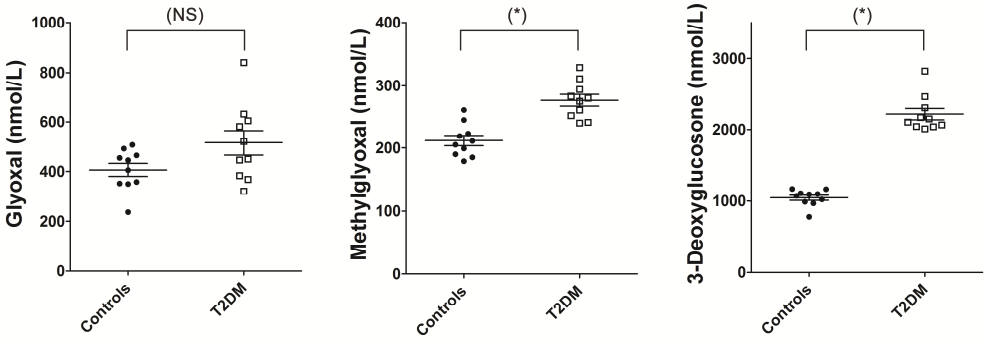

Figure 6.5 Plasma GO, MGO and 3-DG concentrations as measured in non-diabetic controls vs T2DM. Data presented as mean ± SEM (* = p<0.05, NS=not significant).

Discussion

We describe here a rapid, sensitive, precise and accurate method based on stable isotope dilution UPLC MS/MS for the quantification of GO, MGO and 3-DG in blood and plasma. With the use of this method, we found that plasma levels of GO, MGO and 3-DG were increased in T2DM as compared with non-diabetic controls.

Many techniques have been used to quantify GO, MGO and 3-DG. However, several disadvantages of these techniques were noticed, such as large sample volume[1,8,11,13], elaborate LLE or SPE steps[1,2,8,11,16,17] and long run-times[2,12,15]. Moreover, the use of non-stable isotope internal standards[1,5,7,8,17,20] may cause large uncertainties in quantifying analytes using electrospray ionization UPLC MS/MS due to matrix effects[7,8]. So far, the absolute concentrations of GO, MGO and 3-DG differ between studies[5,6,13,21], most likely due to different sample preparation (e.g. deproteinization, duration of derivatisation and calibration) and/or sample handling during storage and the method of detection. In the current study we indeed found large differences in plasma α-oxoaldehyde levels as a result of sample handling during storage.

We present a rapid, sensitive and accurate method for quantifying GO, MGO and 3-DG in plasma. With a sample volume of only 25 µl and a run-to-run time of 8 minutes this method is very suitable for analyzing GO, MGO and 3-DG in large cohort studies. Furthermore, the use of stable isotope internal standards is an improvement over mass spectrometry methods described in literature so far.

GO, MGO and 3DG are very reactive α-oxoaldehydes which can react very fast with proteins to AGEs. In addition to the reaction of these α-oxoaldehydes with proteins, these α-oxoaldehydes can also be formed during sample preparation. Therefore, variations in sample treatment and the interval between the blood draw and sample analysis can substantially influence the free concentrations of these α-oxoaldehydes. We found a progressive increase of MGO and GO in blood drawn in NaF tubes as stored at room temperature. The corresponding plasma also showed an increase of the MGO concentration in time. NaF is used in blood glucose measurements as an inhibitor of glycolysis. Fluoride acts primarily by inhibiting enolase in the glycolytic pathway[22] and prevents the formation of pyruvate out of glucose. Since α-oxoaldehydes can be formed during glycolysis[21], we anticipated α-oxoaldehydes to remain constant in time during storage in NaF tubes. However, this was not the case for MGO and GO. Foregoing explanation for this increase of GO and MGO by NaF is that the inhibition of enolase may lead to the accumulation of precursors for GO and MGO, i.e. dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, which may lead to an increase of GO and MGO during storage in EDTA + NaF tubes. Indeed, this increase in MGO was not noted in EDTA tubes and supported that the effect of fluoride on MGO is due to blockage of the glycolysis at the level of enolase. Because of the above mentioned effect, EDTA + NaF tubes should be avoided and EDTA tubes are preferable.

For the quantification of α-oxoaldehydes in blood, we have found that precipitation of proteins by PCA within 30 minutes after blood withdrawal is necessary. Under this condition, no change in the concentration of MGO and GO and 3-DG was noticed.
For the quantification of α-oxoaldehydes in EDTA plasma, we found that the concentration of α-oxoaldehyde in plasma remained stable for at least 60 minutes without protein precipitation. However, a direct precipitation of plasma proteins by PCA stabilized α-oxoaldehyde levels for at least 2 hours when stored at room temperature and this condition is preferable. Taken together, for quantification of α-oxoaldehydes in blood and plasma, EDTA tubes and a direct PCA precipitation is recommended.
When using EDTA plasma, we found increased levels of α-oxoaldehydes in T2DM as compared to non-diabetic controls which is in reasonable agreement with data described in literature[5,6,13,21]. Nevertheless, absolute concentrations differ between studies; Plasma concentrations as measured in non-diabetic controls varied for MGO from 96 to 652 nmol/L[1,5,6,13,21] and for GO from 328 to 1154 nmol/L[1,5] and the concentration of 3-DG was reported to be 160 nmol/L[5]. In our measurements we found plasma concentrations for MGO, GO and 3-DG of 212 nmol/L, 406 nmol/L and 1046 nmol/L, respectively. The concentration differences of GO, MGO and 3-DG, as found in different studies, are most likely due to sample preparation and sample handling during storage.
In conclusion, for the quantification of α -oxoaldehydes in blood and plasma samples, we recommend EDTA as anticoagulant, a direct protein precipitation of the samples and the use of stable isotope dilution UPLC MS/MS as the method of detection.

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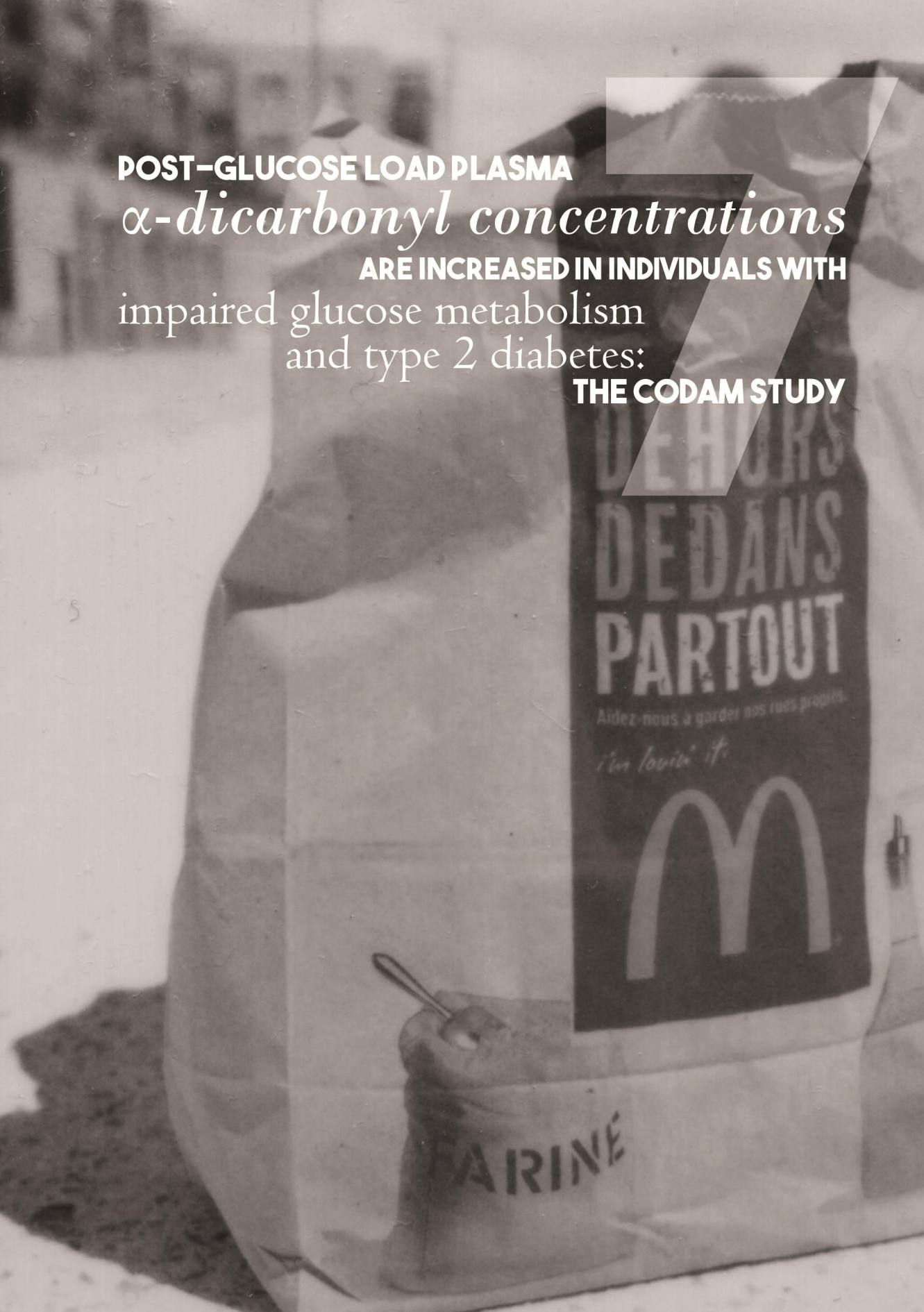

7

POST-GLUCOSE LOAD PLASMA *α-dicarbonyl concentrations* ARE INCREASED IN INDIVIDUALS WITH impaired glucose metabolism and type 2 diabetes: THE CODAM STUDY

7

Post-glucose load plasma α-dicarbonyl concentrations are increased in individuals with impaired glucose metabolism and type 2 diabetes: The CODAM study

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Abstract

Objective:
There is increasing evidence that postprandial glucose excursions play an important role in the development of vascular complications. The underlying mechanism is unknown, but glucose-derived formation of reactive α-dicarbonyl compounds may explain why acute hyperglycemia leads to increased risk for diabetic complications. In the current study, we investigated whether α-dicarbonyls are increased after a glucose load in individuals without and with impaired glucose metabolism (IGM) and type 2 diabetes.

Research Design and Methods:
Cross-sectional, linear analyses were performed in the Cohort study on Diabetes and Atherosclerosis Maastricht (CODAM, n=574, 61% men, 60 years old). Individuals with normal glucose metabolism (n=279), IGM (n=120) and type 2 diabetes (n=92) who had complete data on an oral glucose tolerance test (OGTT) and were not on insulin treatment were included in the study population. Plasma α-dicarbonyl (methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG)) levels were measured in the fasting state and in samples of the OGTT by UPLC-MS/MS.

Results:
The presence of both IGM and type 2 diabetes was significantly associated with higher α-dicarbonyl incremental area under the curves (iAUC), as calculated from the OGTT (for IGM: MGO β=0.190, 95% CI=0.106-0.274; GO β=0.287, 95% CI=0.172-0.401; 3-DG β=0.285, 95% CI=0.221-0.349; for type 2 diabetes: MGO β=0.293, 95% CI=0.180-0.405; GO β=0.536, 95% CI=0.382-0.689; 3-DG β=0.542, 95% CI=0.456-0.628). Adjustment for glucose iAUC attenuated these associations. iAUCs of the α-dicarbonyls correlated highly with glucose iAUC, but not with fasting glucose levels and HbA1C.

Conclusions:
The increased levels of α-dicarbonyls during an OGTT in individuals with IGM and type 2 diabetes underline the potential importance of α-dicarbonyl stress as a candidate to explain the increased risk of diabetic complications in individuals with postprandial hyperglycemia.

Introduction

Type 2 diabetes and impaired glucose metabolism (IGM) are associated with the development of microvascular complications and cardiovascular disease (CVD)[1]. Prospective studies in diabetes have shown a strong correlation between mean glucose levels, as reflected by HbA1C, and diabetic complications. However, in recent years, postprandial glucose excursions, rather than fasting glucose concentrations or mean glucose levels, have increasingly been recognized as a contributing factor to the increased risk of vascular complications[2,3]. Controlled clinical trials, such as the Diabetes Control and Complications Trial (DCCT) in type 1 diabetes[4], and the United Kingdom Prospective Diabetes Study (UKPDS) in type 2 diabetes[5], have established that intensive therapies which reduce HbA1C levels, reduce and delay the development and progression of diabetes-related long-term vascular complications. Post-trial analyses of the DCCT revealed that post-meal glucose levels contribute more to HbA1C¬ than fasting plasma levels[6], suggesting that postprandial hyperglycemic spikes may accelerate the onset of diabetic complications. Moreover, many epidemiological data support this concept, showing that glucose levels after an oral glucose tolerance test (OGTT) are an independent risk factor for CVD, while fasting glucose levels are not or less so[2,7-11]. In addition, postprandial glucose in type 2 diabetes predicts myocardial infarctions[12] and is a risk factor for CVD[13]. However, it is largely unknown how these postprandial glucose peaks cause the increased risk for diabetic complications. A possible mechanism through which they have a more damaging effect than high fasting or mean glucose levels might be via the formation of α-dicarbonyls.

The α-dicarbonyls, methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucoseone (3-DG), are mainly formed as glycolytic intermediates by metabolic conversion of glucose. We and others have shown the importance of α-dicarbonyls in the development of nephropathy[14,15], retinopathy[16] and neuropathy[17,18], and in macrovascular complications[19], using rodent models of diabetes.

We hypothesized that the postprandial glucose concentrations, rather than fasting plasma glucose, determine α-dicarbonyl levels. Although Beisswenger et al. already demonstrated, in a small study of 21 individuals with type 1 diabetes, that both MGO and 3-DG plasma levels increase after postprandial glycemic excursions[20], the question whether α-dicarbonyl levels are elevated in individuals with impaired glucose metabolism (IGM) and type 2 diabetes remains unanswered.

In the present study, we investigated, in a large cohort study, whether plasma levels of α-dicarbonyls are elevated in individuals with IGM and type 2 diabetes. Both fasting levels and incremental area under the curves (iAUC) obtained from an OGTT were analyzed.

Research design and methods

Study population

The present study comprises participants from the Cohort study on Diabetes and Atherosclerosis Maastricht (CODAM), which included 574 participants who were selected from the general population as described in detail elsewhere[21]. The CODAM study was designed to investigate cardiovascular and metabolic function, and was enriched for IGM status, as described previously[22]. Current main analyses were performed on 491 participants who underwent a full OGTT and were not on insulin treatment. The study was approved by the local Medical Ethical Committee of Maastricht University Medical Centre and all participants gave written informed consent.

Definition of glucose metabolism status

The glucose tolerance status of the participants was determined by an OGTT. After an overnight fasting period (10-12 hours), study participants underwent a standard 75 g OGTT (82 g dextrose monohydrate, Avebe, The Netherlands) and venous blood was obtained prior to, and at 30, 60 and 120 minutes after the glucose load. Fasting and post-load plasma glucose concentrations (in mmol/L) were measured with a hexokinase glucose-6 phosphate dehydrogenase method (ABX Diagnosis, Montpellier, France). Fasting and 2h post-load glucose concentrations were used to classify the study participants' glucose metabolism status (GMS) according to the World Health Organization (WHO) criteria. Briefly, individuals were classified as having normal glucose metabolism (NGM) when they had normal fasting (<6.1 mmol/L) and 2h post-load (<7.8 mmol/L) glucose concentrations. Individuals with impaired fasting glucose (6.1-7.0 mmol/L), impaired 2h post-load glucose levels (7.8-11.1 mmol/L), or both were classified as having IGM. When individuals had high fasting plasma glucose levels (>7.0 mmol/L) and/or high 2h post-load glucose levels (>11.1 mmol/L), or when they used glucose-lowering medication or insulin, they were classified as having type 2 diabetes[22]. Individuals with known type 2 diabetes, or those with fasting glucose levels >8.5 mmol/L were excluded from undergoing an OGTT.

Measurements of plasma α-dicarbonyls

Plasma levels of α-dicarbonyls were measured in EDTA plasma samples from the OGTT at baseline and 30, 60 and 120 minutes after the glucose load. Blood samples were collected in EDTA tubes which were stored on ice prior to blood sampling to ensure rapid cooling of the blood.

After withdrawal of the blood sample, tubes were stored on ice immediately, and were spun within two hours at 3000 rpm, 4°C. Plasma samples have been stored at -80°C until analysis. Reversed phase ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to analyze the plasma samples for MGO, GO and 3-DG, as described previously[23]. The inter-assay variations for MGO, GO and 3-DG were 4.3, 5.1 and 2.2%, respectively. Current analyses were performed with fasting α-dicarbonyl levels and OGTT iAUCs.

Calculation of the OGTT incremental area under the curve

The area under the curve (AUC) for the OGTT levels of the α-dicarbonyls and glucose was calculated according to the trapezoidal method[24], where baseline (fasting) levels were subtracted from each individual data point to specify the post-glucose load increases. These data are referred to as the iAUC.

Covariates

Waist circumference and prior CVD were assessed as described previously[25]. Questionnaires were used to asses smoking behavior (never, ever or current smoker) and use of medication (lipid-, glucose- and blood pressure-lowering medication). Plasma creatinine levels were measured with the Jaffé diagnostic test (Roche Diagnostics, Mannheim, Germany) and the estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation[26]. Systolic blood pressures were measured in the brachial arteries and twice in both the tibialis posterior and dorsalis pedis arteries of the lower extremities with a standard Doppler device (Mini Dopplex D900, Huntleigh Diagnostics Ltd, Harmelen, The Netherlands)[27]. Total and HDL-cholesterol and triglycerides were measured in EDTA plasma by enzymatic methods (Roche Diagnostics, Mannheim, Germany)[27].

Statistical analyses

The general characteristics of the study population were compared across the three groups of GMS. Continuous variables were analyzed with one-way ANOVA and discrete variables were analyzed with chi-square tests. Fasting levels and iAUCs from the OGTT were normally distributed for all α-dicarbonyls. To increase statistical power, missing values on HbA1C (3.7% missings), eGFR (0.8% missings) and BMI (0.2% missings) were imputed by creating 5 datasets using multiple imputation. Skewed variables (fasting plasma glucose, fasting plasma insulin, HOMA-IR and triglycerides) were loge transformed prior to analyses.

Two-way repeated measures ANOVA with Bonferroni correction was used to compare the curves of the α–dicarbonyls during the OGTT between NGM, IGM and type 2 diabetes over time. Fasting levels and iAUCs of the α-dicarbonyls were compared between the groups using one-way ANOVA with Bonferroni correction. Multiple linear regression analysis was used to investigate potential influence of confounding factors on the association between GMS and α-dicarbonyl levels. The main independent variables were GMS, analyzed as dummies for IGM and type 2 diabetes, fasting glucose levels, iAUC glucose and HbA1C, and the main dependent variables were fasting levels and iAUCs of the α-dicarbonyls. Data are presented as standardized regression coefficients (β). Analyses were initially adjusted for age and sex, and for analyses with fasting glucose levels, iAUC glucose and HbA1C, also for GMS (model 1). Analyses were further adjusted for smoking status, eGFR, loge triglycerides, total-to-HDL cholesterol ratio, waist circumference, prior CVD, systolic blood pressure and use of medication (lipid-, glucose- and blood pressure-lowering medication) (model 2). Finally, analyses for GMS were additionally adjusted for glucose (fasting or iAUC glucose, appropriate to the α-dicarbonyl measure), as a potential source of α-dicarbonyl formation (model 3), and to investigate to which extent iAUCs were dependent on fasting α-dicarbonyl levels, model 2 was additionally adjusted for fasting plasma levels of the appropriate α-dicarbonyl (model 4). A p-value of <0.05 was considered statistically significant.

As increased levels of α-dicarbonyls can be a result of both increased formation as well as decreased detoxification, we investigated whether individuals with IGM or type 2 diabetes had a decreased capacity to detoxify α-dicarbonyls. To test this, we analyzed whether the association between post-load glucose excursions and α-dicarbonyl iAUCs differed between individuals with NGM, IGM and type 2 diabetes. To this end, the interaction terms IGM*iAUC glucose and type 2 diabetes*iAUC glucose were added to the linear regression model (adjusted for covariates in model 2). $p_{interaction}<0.1$ was considered statistically significant. Significant p-values for interaction would indicate a different detoxification capacity of α-dicarbonyls in individuals with IGM or type 2 diabetes, for the same change in glucose concentration.

All statistical analyses were performed with IBM SPSS Statistics Software, version 20 (IBM Corporation, Armonk, New York).

Results

General characteristics of the study population stratified according to GMS are shown in table 7.1. Overall, participants with type 2 diabetes were slightly older, had a higher BMI, higher blood pressure, lower HDL-cholesterol and more often CVD and microalbuminuria. Curves of the OGTT in figure 7.1 demonstrate increased levels of glucose and MGO, GO and 3-DG after the glucose load. Plasma glucose levels were approximately 5,000-fold higher compared to the plasma α-dicarbonyl levels.

Univariate associations between GMS and both fasting plasma α-dicarbonyls and the OGTT iAUC of the plasma α-dicarbonyls

When we analyzed the fasting α-dicarbonyl levels, we found approximately 1.1-fold higher plasma concentrations of MGO in individuals with type 2 diabetes, but not with IGM (table 7.1, p<0.001), as compared to NGM. Fasting levels of 3-DG were increased in both individuals with IGM and type 2 diabetes compared to those with NGM (table 7.1, both p<0.001). GO levels at baseline did not differ between the groups (table 7.1).

When we additionally included individuals with known type 2 diabetes who were not allowed to undergo an OGTT, we observed significantly higher fasting levels of plasma α-dicarbonyls, compared to levels from those who did undergo the OGTT (figure 7.1).

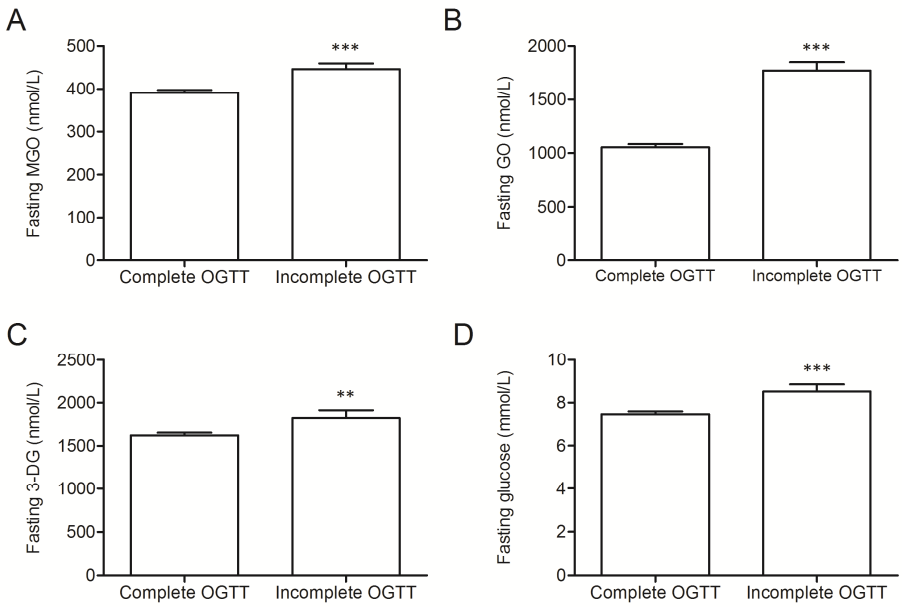

Figure 7.1. Fasting plasma α-dicarbonyl levels of individuals without and with a complete OGTT. Fasting plasma levels of (A) MGO, (B) GO, (C) 3-DG and (D) glucose in individuals who underwent a complete OGTT and individuals who were not able to undergo a complete OGTT. Analyses were performed in all individuals with type 2 diabetes who were not on insulin treatment. Data are shown as means ± standard error of the means. Differences between the two groups were tested with an independent samples t-test. **p<0.01 and ***p<0.001. MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; OGTT, oral glucose tolerance test.

Table 7.1. General characteristics of the CODAM study population stratified according to glucose metabolism status.

	NGM (n = 279)	IGM (n = 120)	Type 2 diabetes (n = 92)	P_{trend}
Age (years)	58.8 ± 7.4	59.9 ± 6.7	60.4 ± 6.2	0.056
Sex (% men)	58.8	60.0	64.1	0.386
BMI (kg/m^2)	27.5 ± 3.9	29.0 ± 4.3	30.1 ± 4.5	<0.001
Waist (cm)	96.0 ± 10.9	100.9 ± 11.9	104.2 ± 11.5	<0.001
HbA_{1C} (%)	5.6 ± 0.4	5.8 ± 0.4	6.6 ± 0.9	<0.001
HbA_{1C} (mmol/mol)	38.0 ± 4.8	40.3 ± 4.7	48.4 ± 9.9	<0.001
Fasting plasma MGO (nmol/L)	350 ± 71	353 ± 57	392 ± 72	<0.001
Fasting plasma GO (nmol/L)	1087 ± 287	1107 ± 279	1057 ± 280	0.442
Fasting plasma 3-DG (nmol/L)	1102 ± 156	1191 ± 136	1619 ± 300	<0.001
Fasting plasma glucose (mmol/L)	5.3 [5.0-5.5]	6.0 [5.5-6.3]	7.3 [6.9-8.2]	<0.001
Fasting plasma insulin (pmol/L)	52.0 [41.0-69.0]	67.0 [46.0-100.0]	85.5 [56.5-124.8]	<0.001
HOMA-IR	0.98 [0.78-1.29]	1.30 [0.89-1.91]	1.72 [1.14-2.49]	<0.001
Glucose-lowering medication (%)	0.0	2.5	33.7	<0.001
Systolic blood pressure (mmHg)	135.6 ± 17.5	143.8 ± 19.3	147.9 ± 18.6	<0.001
Diastolic blood pressure (mmHg)	80.1 ± 8.1	84.1 ± 9.8	85.4 ± 10.3	<0.001
Mean arterial pressure (mmHg)	98.6 ± 10.3	104.0 ± 12.0	106.2 ± 11.5	<0.001
Anti-hypertensive medication (%)	28.3	43.3	51.1	<0.001
Prior CVD (%)	22.9	27.5	37.0	0.009
Ex-smokers (%)	46.6	53.3	59.8	0.072
Current smokers (%)	19.7	19.2	18.5	0.791
eGFR (mL/min/1.73m^2)	90.8 ± 11.7	91.5 ± 11.6	92.3 ± 13.5	0.310
Microalbuminuria (%)	3.9	4.2	9.8	0.048
Macroalbuminuria (%)	0.4	0.8	2.2	0.105
Total cholesterol (mmol/L)	5.2 ± 0.9	5.3 ± 0.9	5.4 ± 1.1	0.058
HDL cholesterol (mmol/L)	1.3 ± 0.4	1.2 ± 0.3	1.1 ± 0.3	<0.001
LDL cholesterol (mmol/L)	3.4 ± 0.9	3.3 ± 0.9	3.2 ± 1.1	0.309
Triglycerides (mmol/L)	1.2 [0.9-1.6]	1.6 [1.1-2.2]	2.0 [1.3-2.6]	<0.001
Lipid-lowering medication (%)	15.1	20.0	19.6	0.221

Data are presented as means ± standard deviations, medians (interquartile range), or percentages, as appropriate. 3-DG, 3-deoxyglucosone; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; GO, glyoxal; HbA1C, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment insulin resistance; LDL, low-density lipoprotein; MGO, methylglyoxal. Linear trend was tested with one-way ANOVA for continuous variables and with chi-square for discrete variables, as appropriate.

iAUCs for the a-dicarbonyls MGO, GO, and 3-DG were significantly higher (1.6-, 2.3- and 1.8-fold change, respectively) in participants with IGM (all p<0.001) and were even higher (2.1-, 3.4-, and 2.6-fold change, respectively) in those with type 2 diabetes (all p<0.001) (figure 7.2B, D, and F) compared with NGM. For glucose, both fasting levels and iAUC of the OGTT were increased in individuals with IGM and type 2 diabetes (table 7.1 and figure 7.2H) (all p<0.001).

Multivariate associations between GMS and fasting plasma α-dicarbonyl levels

The presence of IGM was not associated with higher fasting plasma levels of MGO and GO after adjustment for age and sex (table 7.2, model 1). Fasting plasma 3-DG levels in individuals with IGM were 0.255 standard deviations higher than in those with NGM (β = 0.255; 95% CI: 0.136-0.375; p<0.001). Additional adjustment for smoking, eGFR, triglycerides, total-to-HDL cholesterol ratio, waist circumference, medication, prior CVD and systolic blood pressure did not materially change this association (table 7.2, model 2). Addition of fasting glucose levels to the regression model completely attenuated the association between the presence of IGM and higher fasting levels of 3-DG (table 7.2, model 3).

In contrast, the presence of type 2 diabetes was associated with higher fasting levels of both MGO (β = 0.542; 95% CI: 0.322-0.761; p<0.001) and 3-DG (β = 1.517; 95% CI: 1.385-1.649; p<0.001) after adjustment for age and sex, as compared to NGM. After additional adjustment for multiple covariates in model 2, associations between the presence of type 2 diabetes and higher fasting levels of MGO (β = 0.492; 95% CI: 0.216-0.769; p<0.001) and 3-DG (β = 1.376; 95% CI: 1.211-1.542; p<0.001) were largely unchanged. Further adjustment for fasting glucose levels resulted in an 82% attenuation of the association between type 2 diabetes and fasting MGO levels (table 7.2, model 3). In line, the association between type 2 diabetes and fasting 3-DG levels was attenuated by 61%, but remained statistically significant (table 7.2, model 3). Also after adjustment for potential confounders, we found no associations between either IGM or type 2 diabetes and fasting GO levels.

Multivariate associations between GMS and iAUCs of α-dicarbonyls

In age- and sex-adjusted analyses, the presence of IGM was associated with significantly higher iAUCs for all three α-dicarbonyls (MGO: β=0.203; 95% CI: 0.123-0.283; p<0.001, GO: β=0.268; 95% CI: 0.160-0.377; p<0.001 and 3-DG: β=0.319; 95% CI: 0.257-0.382; p<0.001), as compared to NGM. For type 2 diabetes, the associations with higher iAUCs of all three α-dicarbonyls appeared to be even stronger (MGO: β=0.359; 95% CI: 0.271-0.448; p<0.001, GO: β=0.486; 95% CI: 0.366-0.606; p<0.001 and 3-DG: β=0.645; 95% CI: 0.576-0.714; p<0.001). After further adjustment for the covariates in model 2, both IGM and type 2 diabetes remained significantly associated with higher iAUCs of all three α-dicarbonyls (table 7.2, model 2), compared to iAUCs of individuals with NGM. However, when analyses were further adjusted for the iAUC from glucose, the associations of both IGM and type 2 diabetes with MGO and 3-DG iAUCs disappeared completely (table 7.2, model 3).

Table 7.2. Associations between glucose metabolism status and fasting and iAUC measures of plasma α-dicarbonyls during an OGTT

Determinant	Model	IGM (vs NGM)			Type 2 diabetes (vs NGM)		
		β	95% CI	p-value	β	95% CI	p-value
Fasting MGO	1	0.011	-0.188-0.209	0.917	0.542	0.322-0.761	<0.001
	2	-0.019	-0.226-0.188	0.855	0.492	0.216-0.769	<0.001
	3	-0.129	-0.343-0.085	0.236	0.089	-0.268-0.447	0.624
Fasting GO	1	0.030	-0.142-0.202	0.735	-0.112	-0.303-0.078	0.247
	2	0.063	-0.114-0.240	0.486	-0.088	-0.326-0.149	0.465
	3	0.013	-0.172-0.198	0.891	-0.272	-0.581-0.037	0.085
Fasting 3-DG	1	0.255	0.136-0.375	<0.001	1.517	1.385-1.649	<0.001
	2	0.216	0.092-0.341	<0.001	1.376	1.211-1.542	<0.001
	3	-0.013	-0.122-0.096	0.815	0.536	0.354-0.718	<0.001
iAUC MGO	1	0.203	0.123-0.283	<0.001	0.359	0.271-0.448	<0.001
	2	0.190	0.106-0.274	<0.001	0.293	0.180-0.405	<0.001
	3	0.044	-0.043-0.130	0.321	-0.008	-0.137-0.120	0.897
	4	0.187	0.108-0.266	<0.001	0.362	0.255-0.469	<0.001
iAUC GO	1	0.268	0.160-0.377	<0.001	0.486	0.366-0.606	<0.001
	2	0.287	0.172-0.401	<0.001	0.536	0.382-0.689	<0.001
	3	0.114	-0.006-0.234	0.064	0.180	0.001-0.358	0.049
	4	0.302	0.196-0.408	<0.001	0.514	0.372-0.656	<0.001
iAUC 3-DG	1	0.319	0.257-0.382	<0.001	0.645	0.576-0.714	<0.001
	2	0.285	0.221-0.349	<0.001	0.542	0.456-0.628	<0.001
	3	0.030	-0.013-0.072	0.176	0.015	-0.048-0.079	0.640
	4	0.295	0.231-0.360	<0.001	0.607	0.500-0.714	<0.001

Data were analyzed using linear regression analysis. The standardized regression coefficient β represents the increase of α-dicarbonyl concentrations expressed in standard deviations, compared to NGM. MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; NGM, normal glucose metabolism; IGM, impaired glucose metabolism; CI, confidence interval; iAUC, incremental area under the curve.
Model 1: adjusted for age + sex;
Model 2: model 1 + smoking + eGFR + triglycerides + total-to-HDL cholesterol ratio + waist circumference + prior CVD + systolic blood pressure + medication (lipid-lowering, glucose-lowering and blood pressure-lowering medication);
Model 3: model 2 + glucose (fasting glucose for fasting α-dicarbonyls and iAUC glucose for iAUC α-dicarbonyls);
Model 4: model 2 + fasting levels of corresponding α-dicarbonyls.

Similarly, although the association between type 2 diabetes and higher iAUC of GO (β=0.180; 95% CI: 0.001-0.358; p=0.049) remained significant, it was also attenuated by 66%. Adjustment for fasting levels of the appropriate α-dicarbonyls in model 4 did not change the strength of the association compared to model 2 (table 7.3, model 4 vs. 2).
Since the associations between IGM and type 2 diabetes and higher α-dicarbonyl iAUCs almost completely disappeared after adjustment for glucose, we hypothesized that the higher iAUCs of MGO, GO and 3-DG were a direct result of higher iAUCs of glucose in IGM and type 2 diabetes, rather than a decreased capacity to detoxify α-dicarbonyls.

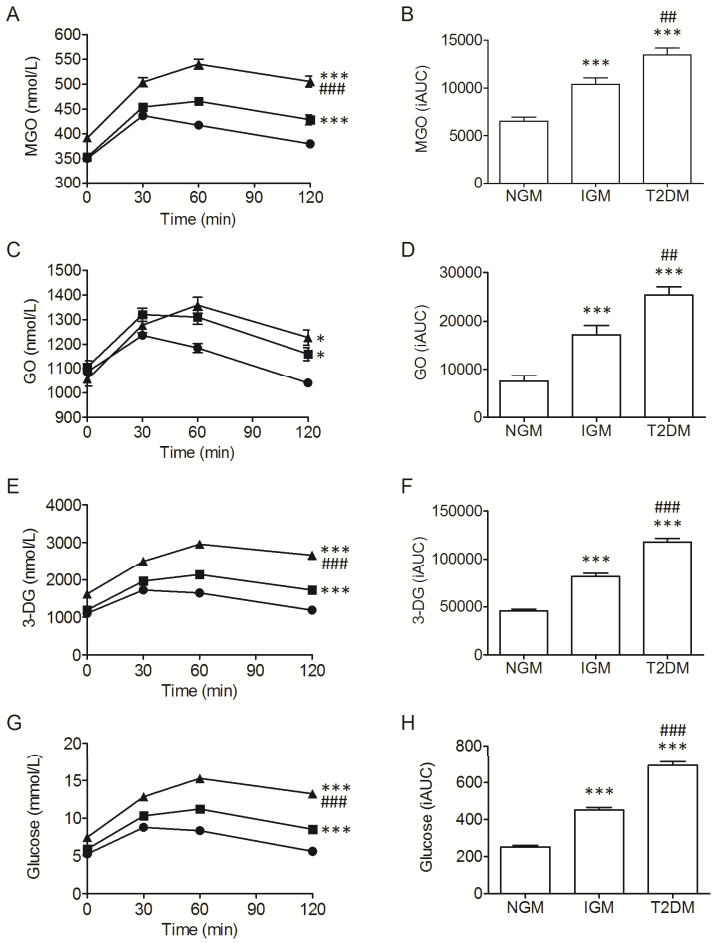

Figure 7.2. Plasma α-dicarbonyl and glucose levels during an oral glucose tolerance test. (A) shows MGO levels during an OGTT over time and (B) demonstrates these data calculated as an iAUC. (C-D) shows the same for GO levels, (E-F) for 3-DG levels and (G-H) for glucose levels. Data are shown as means ± standard error of the means. Circles = NGM, squares = IGM and triangles = type 2 diabetes. Differences in OGTT curves between the groups of GMS were tested with repeated measures two-way ANOVA with Bonferroni correction. Differences in iAUCs between the groups were tested with one-way ANOVA with Bonferroni correction. ***p<0.001, *p<0.05 compared to NGM and ###p<0.001, ##p<0.01 compared to IGM. MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; OGTT, oral glucose tolerance test; iAUC, incremental area under the curve; NGM, normal glucose metabolism; IGM, impaired glucose metabolism.

Indeed, when adjusted for all covariates in model 2, the glucose iAUC was strongly associated with the iAUC of all three α-dicarbonyls (table 7.3; MGO: β=0.195; 95% CI: 0.148-0.243; p<0.001, GO: β=0.231; 95% CI: 0.165-0.297; p<0.001 and 3-DG: β=0.342; 95% CI: 0.318-0.365; p<0.001). These associations did not differ between NGM, IGM or type 2 diabetes for all three α-dicarbonyls (pinteraction>0.1). Overall, associations between the iAUC of glucose and the iAUC of α-dicarbonyls were stronger than associations between HbA1C or fasting plasma glucose and the iAUC of α-dicarbonyls (table 7.3).

Table 7.3. Associations of iAUC glucose, HbA1C and fasting glucose with iAUC of plasma α-dicarbonyls

Determinant	Model	iAUC Glucose			HbA_{1C}			Fasting glucose		
		β	95% CI	p-value	β	95% CI	p-value	β	95% CI	p-value
iAUC MGO	1	0.188	0.143-0.233	<0.001	0.015	-0.026-0.057	0.464	-0.002	-0.069-0.064	0.942
	2	0.195	0.148-0.243	<0.001	0.004	-0.039-0.046	0.865	-0.023	-0.092-0.046	0.521
iAUC GO	1	0.201	0.138-0.264	<0.001	0.020	-0.035-0.075	0.470	-0.059	-0.149-0.030	0.194
	2	0.231	0.165-0.297	<0.001	0.019	-0.039-0.076	0.522	-0.058	-0.152-0.036	0.228
iAUC 3-DG	1	0.337	0.314-0.360	<0.001	0.064	0.028-0.099	0.001	0.032	-0.019-0.084	0.222
	2	0.342	0.318-0.365	<0.001	0.048	0.012-0.085	0.011	0.005	-0.048-0.058	0.857

Data were analyzed using linear regression analysis. The standardized regression coefficient β represents the increase of α-dicarbonyl concentrations expressed in standard deviations per standard deviation increase in iAUC glucose, HbA1C and fasting glucose. MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; CI, confidence interval; iAUC, incremental area under the curve.
Model 1: adjusted for age + sex + glucose metabolism status;
Model 2: model 1 + smoking + eGFR + triglycerides + total-to-HDL cholesterol ratio + waist circumference + prior CVD + systolic blood pressure + medication (lipid-lowering, glucose-lowering and blood pressure-lowering medication).

Conclusions

This study demonstrated that iAUCs of the α-dicarbonyls MGO, GO and 3-DG, as calculated from an OGTT, were higher in individuals with IGM and type 2 diabetes, independently of potential confounders. Fasting plasma levels of α-dicarbonyls were predominantly higher in individuals with type 2 diabetes, although fasting 3-DG levels were also slightly increased in individuals with IGM. After adjustment for glucose, these associations disappeared almost completely for MGO and 3-DG, and to a large, but slightly lesser extent, for GO, indicating that glucose is the major source of α-dicarbonyls. To our knowledge, this is the first time that α-dicarbonyls have been measured in a post-glucose load setting in individuals with NGM, IGM and type 2 diabetes.

In the present study, we found that iAUCs of MGO, GO and 3-DG, as calculated from an OGTT, were higher in individuals with IGM and type 2 diabetes, independently of potential confounders. Our data are in accordance with findings from a previous study by Beisswenger et al, in which they showed increased MGO and 3-DG levels during the postprandial period in patients with type 1 diabetes[20]. As glucose serves as a primary source for the formation of α-dicarbonyls, transient glucose excursions during the postprandial period may give rise to increases of α-dicarbonyl levels, which in turn may induce long-term damage to the vasculature. Indeed, El-Osta et al. demonstrated that even transient exposures to high glucose levels induce persistent changes in cultured endothelial cells, which could be prevented by an overexpression of glyoxalase I (GLO1), the major enzyme detoxifying MGO and GO[28]. We and others demonstrated that increased levels of α-dicarbonyl compounds are directly associated with vascular complications[14,29]. MGO particularly has attracted a lot of attention as a key player in vascular dysfunction, due to its capacity to induce oxidative stress[29], cell death[30] and endothelial dysfunction[14]. Therefore, the observed increased levels of α-dicarbonyls with post-challenge glucose excursions in IGM and type 2 diabetes may link fluctuations in blood glucose levels in these patients with persistent increases in risk of vascular complications and CVD.

The α-dicarbonyls in the plasma can originate from various sources, including in situ formation in the plasma, release from cells and external sources[31]. Due to their rapid increase during the OGTT, the post-load plasma α-dicarbonyl levels most likely originate from intracellular compartments which come directly into contact with plasma glucose. As we found the largest increase in α-dicarbonyls in the individuals with IGM and with type 2 diabetes, it is likely that plasma α-dicarbonyls originate from insulin-independent cells, specifically red blood cells and endothelial cells. Indeed, we demonstrated in human endothelial cells that hyperglycemia produced higher levels of MGO[32] and that α-dicarbonyl levels are much higher in circulating cells than in plasma[23]. We assume that α-dicarbonyl levels after a glucose challenge would increase even further in circulating cells and endothelial cells, as only a small percentage of dicarbonyl compounds leaks into the circulation[23]. To what extent this increase of dicarbonyls in plasma is similar in other tissues that are sensitive to diabetic complications, such as the kidney, nerves, retina and atherosclerotic plaques, remains to be elucidated. Next to release from cells, plasma α-dicarbonyls may also be a result of autoxidation of glucose in the plasma, but it is not known to which extent this process contributes to plasma levels of α-dicarbonyls. It is unlikely however that the iAUCs reflect any autoxidation, as this is a slow process and we observed an increase of α-dicarbonyls already[30] minutes after the glucose load. In addition, dicarbonyl compounds can also originate from exogenous sources[31], for example from the glucose drink we used in this study. However, we previously observed no increased plasma MGO levels in a healthy volunteer within two hours after drinking coffee, a drink with very high levels of MGO (55 µmol/L) but without any glucose. As the glucose drink used for the OGTT only contained 1.6 µmol/L MGO, it is highly unlikely that the low levels of α-dicarbonyls in the glucose drink contribute to their plasma levels after the glucose challenge.

The question remains whether the increased α-dicarbonyl iAUCs are a reflection of decreased detoxification potency in IGM and type 2 diabetes. The glyoxalase system is the major pathway to detoxify MGO and GO[33]. This system consists of the rate-limiting enzyme glyoxalase-1 and glyoxalase-2. These enzymes convert MGO and GO, with the involvement of reduced glutathione, to their end product D-lactate[33]. Several experimental studies have linked the presence of diabetes to dysfunction of the glyoxalase system[34]. However, additional adjustment for glucose in our analyses attenuated the associations between GMS and the iAUC of α-dicarbonyls by 60-97%, suggesting that the elevated post-challenge dicarbonyl levels are the result of increased formation from its substrate glucose. Although dysfunction of α-dicarbonyl detoxification cannot be eliminated as a contributor to increased plasma α-dicarbonyl levels, the lack of interaction between dicarbonyl iAUCs and GMS indicates that detoxification mechanisms are not differently active in any of the three GMS groups.

Our new observation that post-load glucose levels are closely associated with α-dicarbonyl formation is of high clinical relevance because of the current developments in the field of glucose-lowering therapies. Dipeptidyl-peptidase-4 (DPP-4) inhibitors form a very new treatment strategy which has been shown to regulate postprandial glucose concentrations[35]. Whether DPP-4 inhibitors can reduce α-dicarbonyls is unknown. In addition, the bionic pancreas is a state of the art intervention which has been demonstrated to regulate glycemic control very strictly in type 1 diabetes[36], and may prove valuable in type 2 diabetes as well. Directly lowering α-dicarbonyl levels may also be a mechanism to reduce postprandial carbonyl stress and the putative association with vascular damage at long term. One intervention which is currently highly under investigation is pyridoxamine. Pyridoxamine is a chemical scavenger of reactive α-dicarbonyls and has been shown to inhibit formation of AGEs. Several experimental and clinical studies have already demonstrated beneficial effects of pyridoxamine with regard to diabetic microvascular complications[37-39].

The major strengths of this study are that we were able to perform our analyses in a large and well-defined cohort study, and, in addition, we measured plasma α-dicarbonyls with state of the art techniques based on UPLC-MS/MS. There are also a few limitations of this study. First, individuals with known type 2 diabetes did not undergo an OGTT, while they had higher fasting levels of α-dicarbonyl compounds compared to the type 2 diabetes patients who did undergo a full OGTT. Therefore, their α-dicarbonyl levels during the OGTT are expected to increase even more than in newly diagnosed subjects, indicating that our observations may be an underestimation of the true effect in type 2 diabetes. Furthermore, the glucose load in the OGTT is not completely comparable to postprandial glucose excursions. To investigate the effect of postprandial glucose excursions on α-dicarbonyl levels, a mixed meal test needs to be done, although it has been described that the level of glycemia 2h after an OGTT is closely related to the level of glycemia after a standardized meal[40].

Moreover, a major advantage of the OGTT used in this study is that it allowed us to specifically investigate the hypothesis that glucose spikes cause formation of MGO, without confounding of postprandial changes in lipid and protein levels.

In conclusion, we found that significant increases of MGO, GO and 3-DG levels occurred during an OGTT in individuals with IGM and type 2 diabetes in comparison to controls. These increases were strongly associated with post-load glucose excursions. These findings, together with the known harmful biological effects of these α-dicarbonyls, underline the potential importance of dicarbonyl stress as a functional candidate to explain the increased risk of diabetic complications in individuals with postprandial hyperglycemia. Prospective analyses on micro- and macrovascular complications are necessary to associate our current findings with vascular outcome.

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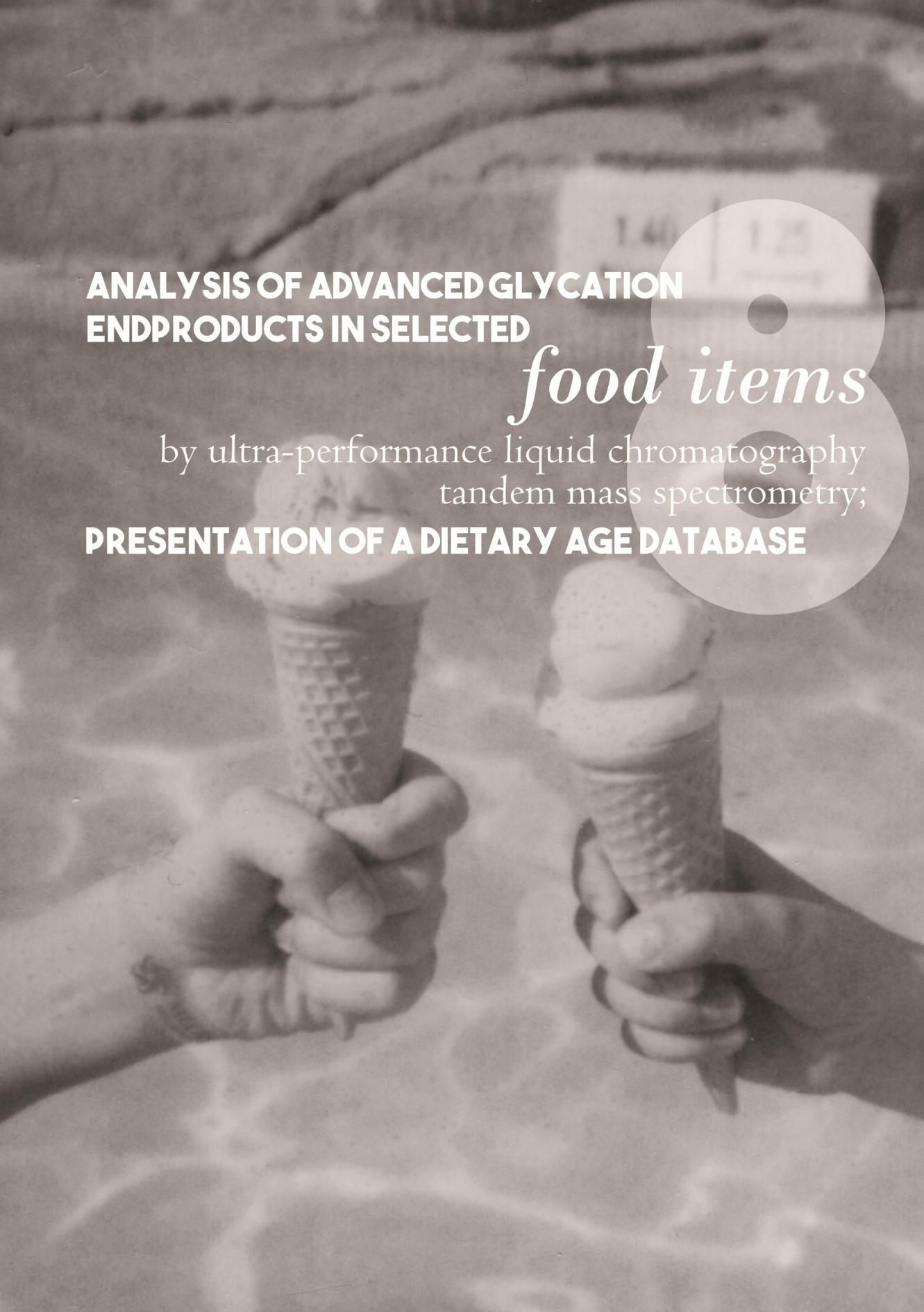

ANALYSIS OF ADVANCED GLYCATION ENDPRODUCTS IN SELECTED *food items* by ultra-performance liquid chromatography tandem mass spectrometry; PRESENTATION OF A DIETARY AGE DATABASE

8

Analysis of advanced glycation endproducts in selected food items by ultra-performance liquid chromatography tandem mass spectrometry; presentation of a dietary AGE database

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Abstract

The aim of this study was to validate an ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) method for the determination of advanced glycation endproducts (AGEs) in food items and to analyze AGEs in a selection of food items commonly consumed in a Western diet. 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 the protein fractions of 190 food items using UPLC-MS/MS. Intra- and inter-day accuracy and precision were 2-29%. The calibration curves showed perfect linearity in water and food matrices. We found the highest AGE levels in high-heat processed nut or grain products, and canned meats. Fruits, vegetables, butter and coffee had the lowest AGE content. The described method proved to be suitable for the quantification of three major AGEs in food items. The presented dietary AGE database opens the possibility to further quantify actual dietary exposure to AGEs and to explore its physiological impact on human health.

Introduction

The non-enzymatic reaction of sugars with proteins, better known as the Maillard or browning reaction, leads to formation of advanced glycation endproducts (AGEs)[1,2]. This reaction was described for the first time in 1912 by the food chemist Louis Camille Maillard[3]. It was not until 1980 that the pathophysiological significance of AGEs emerged in medical science[1,2]. The accumulation of AGEs in the body has been implicated in the pathogenesis of age-related diseases in particular in relation to diabetes, inflammation, neurodegenerative disorders and cardiovascular disease[4-7]. Several mechanisms have been proposed by which AGEs may adversely affect human health, including the binding of AGEs to AGE-receptors, and aberrant cross-linking in the extracellular matrix[8].

The group of AGEs is a heterogeneous family of unavoidable by-products which are endogenously formed by reactive metabolic intermediates derived from sugars and from lipid peroxidation. AGEs formed by sugar-derived carbonyls[9] include N^{ε}-(1-carboxyethyl)lysine (CEL) and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1); AGEs formed by lipid peroxidation and oxidative stress[10,11] include in particular N^{ε}-(carboxymethyl)lysine (CML), a well-known ligand for the receptor for AGEs (RAGE)[12].

The processing and preparation of foods is also susceptible to AGE formation with high temperatures as a potent promoter[13-16]. Animal studies have shown that some of these dietary AGEs are absorbed and that these AGEs, similar to their endogenous counterparts, manifest pathological effects, such as inducing insulin resistance[16-18]. In humans, high- vs. low-CML diets have been shown to increase levels of serum AGEs and serum markers of inflammation and endothelial dysfunction, and to impair flow-mediated dilation and insulin sensitivity[19-23]. However, reliable quantification of AGEs in food, has thus far been a matter of debate[24].

To substantiate whether dietary AGEs pose a risk to human health, a first crucial step is to have access to an accurate method to quantify AGEs and to establish a reliable dietary AGE database. Aside from several reports of small studies, four major studies reported on the AGE content in ~250 to 1500 selected foods and beverages[25-28]. Three of these studies described only the CML content[25-27]. Moreover, three of these studies were based on the determination of AGEs by ELISA[25,26,28], but AGE measurements with immunoassays yield only semi-quantitative results and should thus be interpreted with caution[16,24]. A much better approach for the quantitative determination of specific AGE epitopes in proteins is the use of specific analytical techniques[29]. Several smaller studies have described the detection and quantification of AGEs in food with gas chromatography mass spectrometry (GC-MS)[15,24], high performance liquid chromatography with UV detection (HPLC-UV)[15] or fluorescence detection[30] and with liquid chromatography mass spectrometry (LC-MS)[13,29,31-33]. However, only the quantification of one AGE (CML)[24,31,33] and/or the AGE content of just one food product or food category was reported[13,15,29,32,34].

In the present study we describe a highly sensitive, specific and rapid ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the analysis of CML, CEL and MG-H1 in the protein fraction in food items. We achieved a significant improvement over the methods previously described by using stable isotope labeled internal standards and analysis of three important AGEs. With this validated method, we quantified the concentration of these AGEs in 190 food items which were chosen based upon their habitual and quantitative consumption in the Dutch population.

Materials and Methods

Materials

Boric acid, sodium borohydride, trifluoroacetic acid (TFA) and chloroform were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Butanol and methanol were obtained from Baker (Deventer, the Netherlands). Sodium hydroxide and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Water and acetonitrile were obtained from Biosolve BV (Valkenswaard, the Netherlands). CML (99%), [$^2H_2$]-CML (95.7%), CEL (98.6%), [$^2H_4$]-CEL, MG-H1 (92.6%) and [$^2H_3$]-MG-H1 (98.7%) were obtained from Polypeptide. All other reagents and solvents were of analytical or UPLC/MS grade.

Selection of food items for AGE analysis

Food items for AGE analysis were selected based on the items included in the Dutch cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) 35 and the Dutch National Food Consumption Survey 36. A total of 190 food items were chosen based upon their habitual and frequent consumption by the Dutch population.

Sample preparation protein-bound AGEs

Food items were obtained from local supermarkets and prepared according to the instructions on the manufacturer's label or obtained as prepared food from restaurants (supplemental table, table s8.1). To prevent neo-formation of CML during acid hydrolysis a reduction step with sodium borohydride was used. To check the efficiency of this reduction step a peanut butter sample (~5 mg), a Dutch spiced cake sample (~17 mg) and a milk sample (50 µL), was incubated with 200 µl of 0, 50, 100, 200 and 400 mmol/L sodium borohydride dissolved in 200 mmol/L borate buffer (pH 9.2) for two hours at room temperature. Moreover, the efficiency of the incubation time was investigated by incubating these samples with 100 mmol/L sodium borohydride dissolved in 200 mmol/L borate buffer (pH 9.2) for 0, 2, 4 and 6 hours. Food items containing <20% fat were mixed and subsequently deproteinized with 1000 µl cold (4°C) TFA.

Food items >20% fat were mixed and subsequently deproteinized with a mixture of chloroform:methanol (2:1, v/v) 37. After centrifugation (4300 g, 4°C, 20 min) the supernatant (TFA) or lower phase (chloroform) was carefully removed with a Pasteur pipette. For validation experiments 25 µL of a standard solution (six point calibration curve; 5250-0 nmol/L CML, 6250-0 nmol/L CEL and 14749-0 nmol/L MG-H1) was added. Samples were then hydrolyzed by adding 500 µl 6 N HCl and incubated for 24 hours at 110°C. After hydrolysis, 40 µL hydrolysate and 20 µL internal standard (containing 1432 nmol/L [$^2H_2$]-CML, 1378 nmol/L [$^2H_4$]-CEL and 1322 nmol/L [$^2H_3$]-MG-H1) were mixed in a reaction vial. This mixture was evaporated to dryness under a stream of nitrogen gas at 70°C. To increase sensitivity during electrospray ionization and retention time using reversed phase chromatography, samples were subsequently derivatized with 100 µL 1-butanol:HCl (3:1, v/v) for 90 minutes at 70°C. After derivatisation the samples were evaporated to dryness under nitrogen, redissolved in 300 µL water, mixed and subsequently centrifuged at 20000 g for 20 minutes.

UPLC tandem MS analysis

Derivatized CML, CEL and MG-H1 were analyzed by ultra-performance liquid chromatography (Acquity UPLC, Waters, Milford, USA) and detected in ESI positive multiple reaction monitoring (MRM) mode using a Xevo TQ MS (Waters, Milford, USA). Derivatives were separated on a reversed-phase C18 column (Acquity UPLC BEH C18, 50 x 2.1 mm, 1.7 µm) with a linear gradient of 5 mmol/L ammonia and acetonitril at 48°C. The flow rate was 800 µl/min and the injection volume was 2 µL. Optimal conditions for all parents and daughters were obtained by direct infusion of standard solutions of CML (5250 nmol/L), CEL (6250 nmol/L), MG-H1 (14749 nmol/L), [$^2H_2$]-CML (1432 nmol/L), [$^2H_4$]-CEL (1378 nmol/L) and [$^2H_3$]-MG-H1 (1322 nmol/L). The MRM transitions for CML, CEL and MG-H1 were respectively 317.1>186.1, 331.1>186.1 and 285.1>172.1. The MRM transitions for the internal standards [$^2H_2$]-CML, [$^2H_4$]-CEL, and [$^2H_3$]-MG-H1 were respectively 319.1>186.1, 335.1>190.1 and 288.1>172.1. Electrospray ionization was found optimal at a capillary voltage of 0.5 kV a source temperature of 150°C and a desolvation temperature of 600°C. Quantification of CML, CEL and MG-H1 was performed by calculating the peak area ratio of each unlabeled peak area to the corresponding internal standard peak area. Chromatograms were acquired and processed with Masslynx V4.1 SCN 644 (Waters, Milford, USA).

Method validation

Linearity was determined by adding standard solution of CML, CEL and MG-H1 to water and selected food matrix and subsequently hydrolyzed. To investigate acid stability of CML, CEL and MG-H1 these results were compared to a calibration in water without acid hydrolysis. A six-point calibration curve was therefore prepared for CML (0-5250 nmol/L), CEL (0-6250 nmol/L) and MG-H1 (0-14750 nmol/L). The peak area ratio of the analyte divided by the internal standard area of CML, CEL and MG-H1 multiplied by the concentration of each corresponding internal standard (y, response factor in nmol/L) were plotted as a function of the analyte concentration (x, concentration in nmol/L). The intra-assay variation was determined in three different food items, containing low, medium and high concentration of AGEs, analyzed in one batch during one day (n=8). The inter-assay variation was determined in three different food items divided into batches and analyzed on three different days. To investigate reproducibility, intra-product, intra- and inter-brand variations in different food items a selection of foods, containing both high and low protein content and/or fat percentage, was analyzed in triplicate.
To determine extraction differences between TFA and chloroform:methanol (2:1, v/v) extraction, a milk, white bread and ketchup sample was deproteinized in triplicate, using both extraction fluids. AGE content in these selected food items, contained by both extraction fluids, was compared.

Preparation of minced-meat hot dog

To determine the extent of AGE-formation during preparation of minced-meat hot dog, a standard serving size of minced-meat hot dog was deep fried at a temperature of 190°C. AGE-content was determined in an unbaked minced-meat hot dog (t=0 minutes) and after 2, 4 and 6 minutes of deep-frying.

Results

Reduction of food samples

To prevent neo-formation of CML during acid hydrolysis a reduction step was used. The effectiveness of the reduction step with sodium borohydride was tested with different sodium borohydride concentrations and different incubation times. No further decrease in CML concentration was observed with a reduction step with sodium borohydride concentrations >100 mmol/L for 2 hours at room temperature (figure 8.1A). Under the same conditions, CEL and MG-H1 concentrations were also (figure 8.1A).
No further decrease in CML, CEL and MG-H1 was observed using an incubation time longer than 2 hours (figure 8.1B). Therefore, a reduction step for 2 hours with a concentration of 100 mmol sodium borohydride/L was used for all food samples.

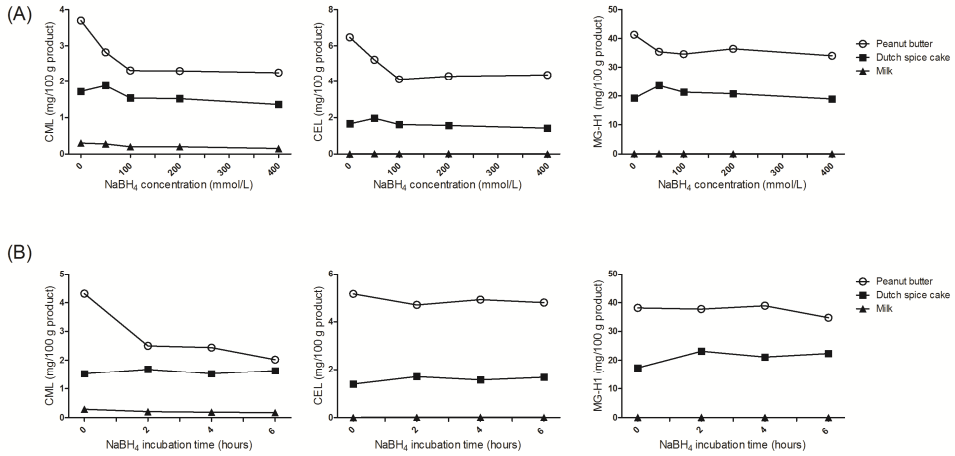

Figure 8.1 The effect of NaBH4 concentration (A) and incubation time (B) on the concentration of CML, CEL and MG-H1 as analyzed in a peanut butter, a Dutch spice cake and a milk sample. Reduction of food samples for 2 hours with 0, 50, 100, 200 and 400 mmol/L NaBH4 (A) and reduction of food samples with a concentration of 100 mmol/L NaBH4 for 0, 2, 4 and 6 hours (B).

Method validation

The calibration curves for CML, CEL and MG-H1 were linear over the described concentration ranges (r^2; 0.90-0.99) in both water and food matrices. Mean slopes (response factor) for CML, CEL and MG-H1 tested in 3 different matrices were 1.13 (CV, 4.4%), 1.65 (CV, 6.1%) and 0.55(CV, 6.8%), respectively (figure 8.2).

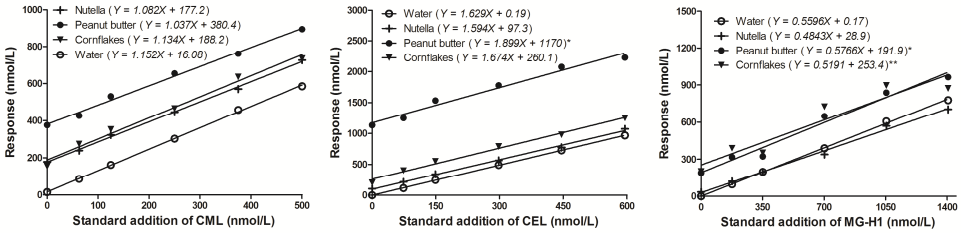

Figure 8.2 Standard addition of CML, CEL and MG-H1 to water, peanut butter, Nutella and Cornflakes.
Linearity was determined by adding standard solution of CML, CEL and MG-H1 to water and selected food matrix (peanut butter, Nutella and Cornflakes) and subsequently hydrolyzed.
The peak area ratio of the analyte/internal standard area of CML, CEL and MG-H1 multiplied by the concentration of each corresponding internal standard (y) were plotted as a function of the analyte concentration (x). Calibration curves showed perfect linearity (r^2>0.99, *r^2 = 0.98 and **r^2 = 0.90).

Impurities coming from the stable isotope internal standard or caused by memory effect of the auto-injector were negligible since calibration curves in water showed no or low y-axis intercepts. Inter-assay variation of CML, CEL and MG-H1, as determined by replicate analysis of a peanut butter, a Mars and a Cornflakes sample on three different days, and intra-assay variation, as determined by replicate analysis (n=8) of these food items on one day, were between 1.7 and 29.1% (table 8.1).

The mean limit of quantification (LOQ), as determined in a peanut butter, a Mars and a Cornflakes sample at a signal-to-noise ratio of 10 (s/N=10) for CML, CEL and MG-H1 were 1, 1 and 25 fmol on column, respectively. These LOQ's were equivalent to 0.02, 0.02 and 0.5 mg of CML, CEL and MG-H1 per 100 g product, respectively.
We applied two different protein precipitation methods for protein pellet collection: 1) Food items containing <20% fat were precipitated with TFA and 2) food items containing >20% fat were precipitated with chloroform:methanol (2:1, v/v)[37].
When we compared both precipitation methods in a milk, white bread and ketchup sample, we found approximately 82% recovery with the chloroform:methanol denaturation as compared to TFA.

Table 8.1 Inter- and intra-assay variation of CML, CEL and MG-H1 as tested in a peanut butter, Mars and Cornflakes sample. Data are expressed as mean AGE mg/100 g product (CV, %).

	CML mean (CV, %)		CEL mean (CV, %)		MG-H1 mean (CV, %)	
	Inter-run (n=3)	Intra-run (n=8)	Inter-run (n=3)	Intra-run (n=8)	Inter-run (n=3)	Intra-run (n=8)
Peanut butter	1.48 (7.0)	1.38 (5.1)	3.84 (4.1)	3.74 (6.7)	18.69 (17.9)	20.39 (25.0)
Mars	0.67 (15.0)	0.70 (4.7)	0.33 (9.8)	0.36 (6.0)	0.14 (16.8)	0.15 (18.3)
Cornflakes	0.72 (7.7)	0.66 (12.8)	0.84 (6.7)	0.79 (16.2)	12.27 (1.7)	12.12 (29.1)

Acid stability of AGEs

The calibration curves for CML, CEL and MG-H1, as tested in water after acid hydrolysis, were compared to the calibration curves without acid hydrolysis. The slope for CML, CEL and MG-H1 as tested in water, without acid hydrolysis, was 1.18, 1.59 and 1.04, respectively. For CML and CEL no differences were observed between the mean slopes of the calibration curve with or without acid hydrolysis. For MG-H1, however, a decrease of the mean slope from 1.03 to 0.56 was observed after acid hydrolysis. Thus, under these standard conditions we found a recovery of MG-H1 of ~55%.

AGE formation during preparation of minced-meat hot dog

To further investigate the formation of AGEs during food heating, minced-meat hot dog was fried at a temperature of 190°C in frying fat. Unbaked minced-meat hot dog contained 0.28, 0.10 and 3.30 mg CML, CEL and MG-H1 per 100 g product, respectively. After 2, 4 and 6 minutes of frying, samples were collected and the concentrations of all three AGEs increased in a time-dependent manner (figure 8.3). After 6 minutes of deep-frying CML, CEL and MG-H1 were increased to 4.82, 11.35 and 33.64 mg per 100 g product, respectively.

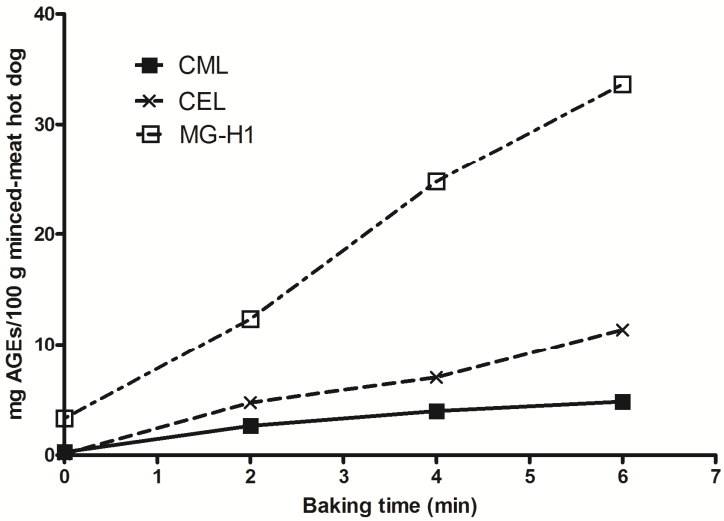

Figure 8.3 Formation of AGEs in deep fried minced-meat hot dog. AGE-formation during preparation of minced-meat hot dog during deep frying at a temperature of 190°C. AGE-content was determined in an unbaked minced-meat hot dog (t=0 minutes) and after 2, 4 and 6 minutes of deep-frying.

AGE content in selected food items

The complete list of AGE levels in the selected food items is presented in table s8.1 (supplemental table). Black pudding, cereals (miniwheats, frosted flakes, cornflakes and puffed wheat), peanut butter, peanuts, rusk and biscuits (with peanuts, speculoos and multigrain), contained high levels of CML, CEL and MG-H1 (supplemental table s8.1). Overall the food items which were prepared by an exposure to heat and long processing times (such as black pudding fried for 16 minutes or red cooked beefstewed for 2.5 hours in wine vinegar) or baked (such as biscuits, cake and knäckebröd) or industrial conservation (such as canned products which were commercially sterilized at 115-130°C for 15 minutes) or products containing chocolate, nuts or grains were high in AGE content.

Food items with high AGE content varied from ~2 to ~5 mg CML, from ~2 to ~7 mg CEL and from ~15 to ~60 mg MG-H1 per 100 g product. Coffee, fruits, vegetables, butter, olive oil and red wine had negligible AGE content. Overall, the food items with a short heat processing time, low protein and/or carbohydrate content or high water content had low or negligible levels of CML, CEL and MG-H1.

Discussion

Here, we describe a sensitive, precise and well-validated UPLC-MS/MS method for the quantification of the AGEs CML, CEL and MG-H1 in a broad range of different food items. We report a database of CML, CEL and MG-H1 in 190 food items. We found high AGE levels in food items which had been prepared by an exposure to heat, industrial conserving or products containing chocolate, nuts or grains, whereas food products with short or no heat processing, low protein and/or carbohydrate content, or high water content had low or negligible AGE content.

The protein-bound AGE concentrations in foods have previously been analyzed by different sample preparation techniques. Depending on the acid stability of the AGE of interest, the protein-bound AGE content was determined by means of hydrochloric acid hydrolysis[14,15,27,33,38] or by enzymatic hydrolysis using a cocktail of proteases[13,29,38]. In our study CML and CEL proved to be acid stable, but MG-H1 showed a reproducible decrease of approximately 45% after acid hydrolysis. Therefore, the MG-H1 content was adjusted for this decrease. This decrease in MG-H1 was in agreement with previously reported data[39], but we found that the order of magnitude was less than the approximately 90% reduction in hydroimidazolones after acid hydrolysis which was previously reported. This difference may be explained by sample preparation differences, such as the addition of nitrogen, phenol and/or mercaptoethanol during acid hydrolysis. With an inter- and intra-assay variation of approximately 10%, linear and reproducible calibration curves in both water and food matrix and a high specificity and sensitivity, the analysis proved to have good validity.

In the present study peanuts, peanut butter, rusk, biscuits, cereals, toast and high-heat-processed meats, such as red cooked beef, canned beef steak and black pudding, had the highest levels of CML, CEL and MG-H1, while coffee, fruits, vegetables, butter, olive oil and red wine had negligible AGE content. For CML these findings were in accordance with data described in literature[27] although absolute levels of CML content differ. Food preparation techniques, such as pasteurization, sterilization, roasting, baking, frying or boiling, could strongly influence the AGE content[13-15,29,33] and therefore comparison of absolute AGE levels between studies is difficult. Moreover, differences between the ingredient profile, sample preparation or quantitative methodology should also be considered.

AGE formation during heat processing, industrial cooking or cooking in sauce, as observed in many studies[13,14,26,27,29,30,33,38], was confirmed in our study. We observed an increase in AGE content in minced-meat hot dog during frying. Moreover, high AGE levels were observed in red cooked beef, which was stewed in wine vinegar sauce. We also found a 5- to 10-fold increase in AGE content in semi-skimmed evaporated milk compared to semi-skimmed milk and these results were in good accordance with Hegele et al.[38] and Assar et al.[33] who observed that milk products which had undergone extensive industrial processing, such as condensed or evaporated milk, were high in AGE content.

In general, high heat treatment or a long treatment time in canning or stewing is most likely responsible for generating elevated AGE levels in food.

So far, three major studies have described the CML content in a large selection of food items[25-27] and one major study investigated four AGEs, including CML, in beverages and foods commonly consumed in Japan[28]. Three of these studies were based on ELISA technique[25,26,28] and one study investigated CML content in foods using UPLC-tandem MS analysis[27].
In the present study we found low or negligible CML content in mayonnaise, butter and olive oil which was in agreement with the findings of Hull et al.[27] using an UPLC-MS/MS technique. Differences in the CML content of a Mars and Bounty candy bar were observed. We found approximately a 2-fold lower CML content in Mars and a 10-fold lower CML content in a Bounty candy bar as compared to the results of Hull et al.[27]. Moreover, we found a 6-fold lower CML content in whole meal bread, Kellog's corn flakes, white rice, parmesan cheese and canned salmon as compared to their results. These differences may be explained by differences in sample preparation or ingredient profile.

Using an ELISA technique, Uribarri et al.[26] found high AGE levels in all products containing fat such as butter, olive oil, cheese and milk products and also in most meats but not in food items that are expected to contain high AGE levels such as biscuits, crackers and cookies. In contrast, we found that most cheese and milk products are low in AGE content (with the exception of evaporated milk). Indeed, previous studies show also inconsistencies in CML content in foods when analyzed by mass spectrometry or ELISA[24,40]. These differences might be caused by an unspecific interference of the lipid matrix with the ELISA[24] since ELISA techniques have a high risk of matrix interference as the antigen-antibody interaction is dependent on the chemical environment[16].

The main advantages of the present study above the previously described studies are the use of a state-of-the-art UPLC-tandem MS technique and the ability to analyze three major AGEs in one run.
To further investigate the relevance of dietary AGEs in disease, the presented reliable and valid analytical method is suitable for the analysis of CML, CEL and MG-H1 in selected food items and is a strong tool to build up an extensive dietary AGE database. The present dietary AGE database will be valuable for designing intervention studies with low and high AGE diets to study the health effect of AGEs in much more detail than has hitherto been possible.

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Appendix 8

Supplemental table

Supplemental table 8

Table s8.1 CML, CEL and MG-H1 content in food (* content in mg/100 g food).

Potatoes

	Food	Specification	CML*	CEL*	MG-H1*
1	Chips	Deep fried 5 minutes	0.14	0.09	1.28
2	Potato-boiled	Boiled, 7 minutes	0.01	0	0.39
3	Potato-fried	Boiled, 7 minutes, fried 6 minutes	0.01	0.01	0.3

Alcoholic and non-alcoholic beverages

	Food	Specification	CML*	CEL*	MG-H1*
4	Coffee		0	0.01	0.24
5	Red wine		0	0	0
6	Fruit juice	Kiwi/Orange	0	0	0.05
7	Cola		0	0	0

Eggs

	Food	Specification	CML*	CEL*	MG-H1*
8	Egg yolk	Boiled, 10 minutes	0.06	0.08	0.08
9	Egg white	Boiled, 10 minutes	0.1	0.07	0.35
10	Fried egg	Fried, 4 minutes	0.42	0.52	4.5

Fruits

	Food	Specification	CML*	CEL*	MG-H1*
11	Kaki		0	0	0
12	Banana		0.01	0	0.05
13	Apple sauce	Light	0	0	0.2
14	Olives	Green	0.12	0.01	1.54

Vegetables

	Food	Specification	CML*	CEL*	MG-H1*
15	Mushroom	Fried, 5 minutes	0.01	0.01	0.01
16	Peas	Boiled	0.05	0.06	2.31
17	Tomato	Fried, 3 minutes	0.07	0.05	0.65
18	Tomato, canned		0.03	0.01	0.11

Savoury bread spreads

	Food	Specification	CML*	CEL*	MG-H1*
19	Peanut butter (n=3)**	Pâte de cacahouètes, 72% peanut	1.91 ± 0.23	5.56 ± 0.31	35.62 ± 2.51
20	Peanut butter (n=3)**	Delvita Erdnuss Crème, 90% peanut	3.11 ± 0.31	6.74 ± 0.45	44.52 ± 2.03
21	Peanut butter (n=3)**	Calvé, 85% peanut	2.63 ± 0.68	6.85 ± 0.68	42.05 ± 4.08
22	Peanut butter (n=8)**	Plus, 90% peanut	1.38 ± 0.07	3.74 ± 0.25	20.39 ± 5.09

Cheese

	Food	Specification	CML*	CEL*	MG-H1*
23	Cheese	Gouda 48+ (32% fat)	0.03	0.01	0.08
24	Cheese	(32% fat)	0.04	0.08	0.25
25	Cheese	Old (35% fat)	0.08	0.22	0.21
26	Parmesan cheese	Parmigiano Reggiano (28% fat)	0.22	0.63	2.76
27	Cheese	(32% fat)	0.15	0.55	0.3
28	Cream cheese	(7% fat)	0.35	0.08	0.49
29	French cheese	Brie, Coeur de Lion, 60+ (32% fat)	0.01	0	0.07
30	French cheese	Camembert Chêne d'argent (21% fat)	0.12	0.3	0.16
31	Cottage cheese		0.05	0.04	0.12
32	Mozzarella		0.07	0.01	0.13
33	Feta cheese		0.08	0.47	1.14

Milk and milk products

	Food	Specification	CML*	CEL*	MG-H1*
34	Milk	Semi-skimmed	0.25	0.02	0.12
35	Milk	Skimmed	0.07	0.01	0.08
36	Milk	Skimmed	0.26	0.02	0.3
37	Milk	Skimmed, microwave 2 min	0.36	0.02	0.38
38	Yoghurt drink	0% fat, raspberry	0.04	0.03	0.12
39	"Kwark"	Curd, skimmed	0.06	0.04	0.26
40	Milk	Whole	0.01	0.01	0.02
41	Evaporated milk		0.66	0.03	0.09
42	Evaporated milk	Skimmed	0.47	0.08	0.38
43	Evaporated milk	Semi-skimmed	2.23	0.12	0.54
44	Whipped cream	35% fat	0	0	0
45	Chocolate milk	Whole	0.38	0.2	0.66
46	Chocolate milk	Semi-skimmed	1.01	0.26	0.98
47	Custard	"Vla", chocolate/vanilla	0.12	0.06	0.43
48	Porridge	Rolled oats	0.02	0	0.06
49	Porridge	Rice	0.03	0.02	0.21
50	Pudding	Raspberry, with strawberry sauce	0.26	0.02	0.33
51	Yogurt	Semi-skimmed, vanilla	0.01	0.01	0.13
52	Ice-cream	Vanilla/Strawberry	0	0	0.03

Soy products and vegetarian products

	Food	Specification	CML*	CEL*	MG-H1*
53	Vegetarian burger	Vegetable burger, fried 5 minutes	0.42	0.6	3.6
54	Tofu	Fried 10 minutes	0.94	0.74	11.87
55	Tempe	Boiled, 10 minutes	0.47	0.29	3.5
56	Tempe	Grilled, 4 minutes	1	0.59	6.87
57	Soy sauce	Ketjap manis	0.01	0.02	0.05

Sugar, sweets and sweet sauces

	Food	Specification	CML*	CEL*	MG-H1*
58	Praline	With orange crème filling	0.41	0.09	0.87
59	Chocolate	White	0.52	0.06	0.42
60	Chocolate	Milk	0.96	0.31	1.33
61	Chocolate	Milk, with hazelnuts	1.33	0.55	3.23
62	Chocolate	Dark	3.51	1.28	3.03
63	Mars (n=8)**		0.70 ± 0.03	0.36 ± 0.02	0.15 ± 0.03
64	Twix		0.93	0.81	5.83
65	Bounty		0.71	0.23	2.16
66	Milky Way		1.68	0.56	0.54
67	Liquorice		0.06	0.02	0.08
68	Candy	Jelly bears	0.03	0.02	0.11
69	Jam	Blackberry	0	0	0
70	"Hagelslag"	Chocolate sprinkles	5.09	2.03	9.33
71	"Hagelslag"	Fruit sprinkles	0	0	0
72	"Stroop"	Apple syrup	0.05	0.02	0.44
73	Chocolate spread		0.9	0.34	3.27

Fats, oils and savoury sauces

	Food	Specification	CML*	CEL*	MG-H1*
74	Peanut sauce	Microwave heated, 0.5 minutes, 800 Watt	0.21	0.33	1.9
75	Peanut sauce	Microwave heated, 1.5 minutes, 800 Watt	0.65	0.95	6.94
76	Peanut sauce	Instant, 90% peanut, 1:1 made up with boiling water	1.29	2.35	14.03
77	Peanut sauce	Pan heated, 500g with 300ml water	0.8	1.39	12.6
78	Tomato sauce	Pan heated	0.04	0.05	0.22
79	Curry sauce	Instant, 1:10 made up with boiling water	0.05	0.01	0.13
80	Cheese-sauce	Instant, 1:6 made up with boiling water	0.06	0.02	0.18
81	Mayonnaise		0.01	0	0.12
82	Frying butter	Liquid, pan heated	0	0	0
83	Butter	Diet (60% fat)	0	0	0
84	Olive oil	Pan heated	0	0	0
85	Ketchup		0.05	0.01	0.05
86	Garlic sauce		0.01	0	0.02

Fish

	Food	Specification	CML*	CEL*	MG-H1*
87	Salmon, canned	In brine	1.17	2.82	10.93
88	Salmon, fried	Fried, 10 minutes	0.41	0.58	1.83
89	Salmon, smoked		0.58	0.22	0.32
90	Herring	Raw	0.06	0.06	0.09
91	Hake	Breaded, oven heated, 10 minutes 200°C	0.38	0.31	0.55
92	Whitefish	Grilled, in soy sauce	0.26	0.29	1.08
93	Prawns	Fried, 5 minutes	0.06	0.07	1.03
94	Tuna, canned	In olive oil	0.35	1.26	3.78
95	Cod	Fillet, fried 8 minutes	0.13	0.11	0.66
96	Steamed fish	Oven heated, 15 minutes 175°C	0.05	0.06	0.11
97	Fish fingers	Oven heated, 15 minutes 175°C	0.3	0.18	4.3

Nuts, seeds and snacks

	Food	Specification	CML*	CEL*	MG-H1*
98	Pistachios	Unprocessed	0.21	0.09	0.32
99	Pecans	Roasted, salted	1.06	1.95	16.63
100	Peanuts	In shell	1.37	2.43	25.71
101	Peanuts	Salted	1.72	3.39	26.59
102	Cocktail nuts	"Borrelnoten" Italian herbs	1.05	0.82	6.82
103	Cocktail nuts	"Borrelnoten" Sateh curry	1.58	2.22	12.34
104	Cocktail nuts	"Borrelnoten" Spicy	0.55	1.13	8.06
105	Potato crisps	Kettle	0.23	0.27	1.63
106	Corn crisps	Vitasia Tikka Masala	0.29	0.11	2.79
107	"Kroket"	Beef filling, deep fried 7,5 minutes	0.41	0.44	4.06
108	Sausage roll	Oven heated, 8 minutes	1.01	0.45	5.26
109	Satay	Pork, with peanut sauce, microwave heated 60 seconds	0.45	0.9	5.7
110	"Frikandel"	Deep fried 7,5 minutes	0.99	1.42	5.07

Mixed dishes

	Food	Specification	CML*	CEL*	MG-H1*
111	Spring roll	Deep fried, with vegetables and meat	0.2	0.07	1.53
112	Pizza	Mozzarella pesto, oven heated 12 minutes 175°C	0.06	0.05	0.95
113	Russian salad	With beef, potato, onion, peas, mayonnaise	0.13	0.03	0.67

Soups

	Food	Specification	CML*	CEL*	MG-H1*
114	Tomato soup, canned	Microwave heated, 1,5 minutes 800 Watt	0	0	0
115	Pea soup	Home made, heated	0.04	0.11	2.23

Meats, meat products and poultry

	Food	Specification	CML*	CEL*	MG-H1*
116	Minced beef (10% fat)	Beef, fried, 8 minutes	0.37	0.44	0.54
117	Red cooked beef (n=3)**	Stewed, 2,5 hours in wine vinegar	2.03 ± 0.45	5.63 ± 1.12	13.48 ± 6.01
118	Minced beef (n=2)**	Steamed, 15 min	0.44 ± 0.11	0.25 ± 0.11	2.06 ± 1.38
119	Minced beef (n=2)**	Fried, 10 minutes	0.51 ± 0.08	0.31 ± 0.10	0.86 ± 0.24
120	Beef steak	Grilled 5 minutes	0.73	0.4	0.9
121	Roast beef	Oven heated, 1 hour 150 C	1.01	0.41	2.09
122	Beef steak (n=3)**	Fried, 4 minutes	0.12 ± 0.08	0.23 ± 0.11	0.34 ± 0.12
123	Beef steak (canned) (n=3)**	Pan heated, 10 minutes	1.07 ± 0.38	5.63 ± 2.01	11.96 ± 2.78
124	Pork, shoulder chops (n=3)**	With garlic, fried 12 minutes	0.15 ± 0.04	0.35 ± 0.17	0.51 ± 0.24
125	Pork (n=3)**	Fried, 3 minutes, then stewed, 40 minutes	0.61 ± 0.12	1.03 ± 0.28	1.71 ± 0.53
126	Pork fillet roulade	Oven heated, 40 min 175 C	0.11	0.15	0.49
127	Minced beef (21% fat)	50/50 beef and pork, fried 5 minutes	0.94	1.68	2.19
128	"Slavink"	Minced beef wrapped in bacon, fried, 20 minutes	0.14	0.09	0.77
129	Sausage	Fried, 13 minutes	0.84	1.63	3.85
130	Fried bacon	Fried	0.72	1.82	3.01
131	Pork	Strips, fried 7 minutes	1.12	1.15	1.9
132	Frankfurter, canned (n=2)**		4.22 ± 0.16	1.01 ± 0.09	4.74 ± 0.68
133	Frankfurter, canned (n=2)**	Oven heated, 15 min 175 C	3.33 ± 0.98	1.09 ± 0.22	4.22 ± 0.67
134	Meat ball		0.83	0.9	4.61
135	Meat ball	Oven heated, 15 min 175 C	1.27	1.91	6.95
136	Roasted pork	Roasted	0.41	0.29	3.55
137	Pork tenderloin (n=3)**	Fried, 16 minutes	0.34 ± 0.25	1.39 ± 1.14	3.23 ± 2.27
138	Gammon steak (n=3)**	Fried, 25 minutes	0.22 ± 0.07	0.89 ± 0.61	1.04 ± 0.34
139	Schnitzel (n=3)**	Non-breaded, fried 6 minutes	0.12 ± 0.05	0.44 ± 0.35	0.23 ± 0.16
140	Rib eye	Fried, 15 minutes	0.27	0.35	0.79
141	Hamburger	Fried, 8 minutes	0.2	0.29	1.58
142	Black pudding	With crushed bacon, fried 16 minutes	4.82	7.71	63.01
143	Lamb, chops (n=3)**	Fried, 6 minutes	0.11 ± 0.03	0.30 ± 0.08	0.66 ± 0.22
144	"Rookworst" (n=2)**	Smoked sausage, beef, heated in hot water	0.98 ± 0.08	0.41 ± 0.18	1.06 ± 0.12
145	"Rookworst" (n=2)**	Smoked sausage, pork, heated in hot water	1.67 ± 1.27	0.46 ± 0.15	2.19 ± 0.27
146	Gammon (boiled)	Cold cut	0.12	0.33	0.37

Meats, meat products and poultry

	Food	Specification	CML*	CEL*	MG-H1*
147	Ham, shoulder (boiled)	Cold cut	0.32	0.66	1.26
148	Steak tartare	Sweet onion	0.08	0.04	0.21
149	Roast beef	Cold cut	0.28	0.41	0.44
150	Salami	Cold cut	1.27	0.58	3.15
151	Salami sausage saveloy	Cold cut	0.54	0.19	2.43
152	Bacon rashers	Cold cut	2.54	0.93	0.71
153	Drumsticks	With skin, oven heated, 40 minutes, 200°C	0.62	0.95	2.21
154	Chicken Wings	With skin, oven heated	0.82	0.78	3.04
155	Ragout (chicken stew)	Pan heated	0.65	0.79	2.93
156	Crusty chicken	Breaded	0.75	0.73	4.05
157	Chicken fillet	Fried, 30 minutes	0.13	0.27	1.41
158	Chicken (n=2)*	Boiled, 30 minutes	0.18 ± 0.03	0.14 ± 0.07	0.65 ± 0.07
159	Chicken (n=2)*	Fried, 10 minutes	0.34 ± 0.02	0.26 ± 0.04	1.16 ± 0.41
160	Chicken	Microwaved, 10 minutes	0.92	1.02	5.16
161	Pâté		0.09	0.07	0.29
162	Liverwurst (n=2)*		0.25 ± 0.14	0.30 ± 0.04	1.59 ± 0.41

Bread

	Food	Specification	CML*	CEL*	MG-H1*
163	Wholemeal bread		0.28	0.21	3.36
164	Brown bread		0.4	0.31	6.01
165	Rye bread	Dark, brand A	0.9	0.09	2.57
166	Rye bread	Dark, brand B	0.58	0.18	2.74
167	Rye bread	Light	0.59	0.18	5.22
168	White bread (n=3)**		0.24 ± 0.02	0.11 ± 0.02	2.55 ± 0.33
169	White bread (n=3)**	Belgian made	0.21 ± 0.05	0.11 ± 0.01	2.48 ± 0.19
170	White bread (n=3)**	German made	0.31 ± 0.01	0.13 ± 0.01	2.09 ± 0.15
171	White bread (n=3)**	Dutch made	0.13 ± 0.02	0.07 ± 0.01	2.18 ± 0.04
172	Currant bread		1.11	0.56	3.35
173	Luxurious breads/buns	Baguette, oven heated, 9 minutes, 200°C	0.22	0.1	2.28
174	Croissant		1.04	1.33	9.89
175	Rusk		1.96	1.44	23.1
176	Knäckebröd		0.96	1.41	13.5
177	Toast (bread)	2½ minutes in toaster	0.52	0.55	4.16
178	Toast (cracker)		2.31	0.76	9.97
179	Baguette, toasted	2½ minutes in toaster	0.23	0.1	3.61
180	White bread without crust	Belgian made	0.21	0.03	1.86
181	Wholemeal bread without crust		0.24	0.05	4.1
182	Pita bread	Oven heated, 15 min, 175°C	0.25	0.06	2.82

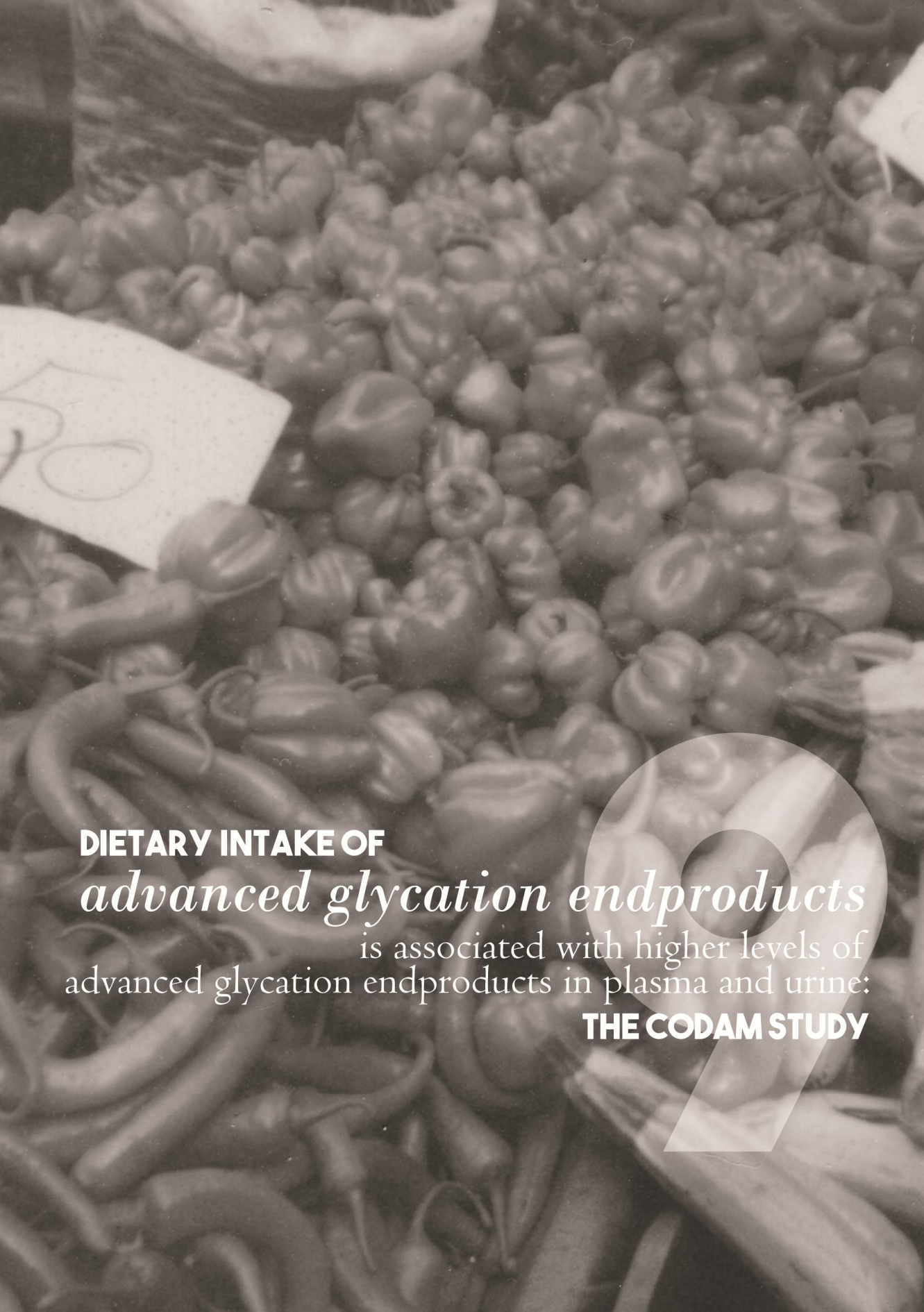
Pastry and biscuits

	Food	Specification	CML*	CEL*	MG-H1*
183	Dutch spiced cake (n=3)**	Dutch made (Batch 1)	0.79 ± 0.05	1.22 ± 0.09	20.31 ± 0.82
184	Dutch spiced cake (n=3)**	Dutch made (Batch 2)	0.91 ± 0.02	1.13 ± 0.27	19.76 ± 1.81
185	Dutch spiced cake (n=3)**	Belgian made (Batch 1)	0.65 ± 0.09	0.84 ± 0.19	12.97 ± 2.42
186	Dutch spiced cake (n=3)**	Belgian made (Batch 2)	0.74 ± 0.17	0.84 ± 0.27	15.87 ± 2.12
187	Dutch spiced cake (n=3)**	German made (Batch 1)	0.79 ± 0.11	0.91 ± 0.08	8.07 ± 0.14
188	Dutch spiced cake (n=3)**	German made (Batch 2)	0.91 ± 0.08	1.24 ± 0.33	10.87 ± 0.78
189	Apfelstrudel	Oven heated 40 minutes, 200°C	0.07	0.05	0.97
190	Apricot pie		0.25	0.47	3.47
191	Cream pie		0.48	0.03	1.14
192	Tompouce		0.27	0.24	2.23
193	Pancake	With mascarpone	0.49	0.17	1.38
194	Cake		1.75	0.23	2.61
195	Syrup waffle	"Stroopwafel"	0.46	0.58	11.31
196	Cake	With almond paste	0.51	0.55	5.57
197	Biscuit	Multigrain	1.33	1.06	19.62
198	Biscuit	With peanuts	2.59	3.42	36.9
199	Biscuit	Speculoos	2.47	1.99	28.31
200	Biscuit	With currants and cherry	1.03	0.48	3.99
201	Rice cracker	With cheese powder	1.13	1.04	11.34
202	Rice cracker	With sea salt	0.91	0.75	8.01

Cereals and cereal products

	Food	Specification	CML*	CEL*	MG-H1*
203	Cereals	Honey loops	1.2	0.7	3.6
204	Cereals	Honey granola	1.04	1.02	9.44
205	Cereals	Crunchy muesli	0.46	0.46	8.61
206	Cereals (n=8)**	Cornflakes	0.66 ± 0.08	0.79 ± 0.13	12.12 ± 3.53
207	Cereals	Puffed wheat	1.27	0.84	15.61
208	Cereals	Frosted flakes	0.63	0.53	25.89
209	Cereals	Rice crispies	0.59	0.47	14.7
210	Cereals	Cooked oats	1.17	0.43	11.31
211	Cereals	Miniwheats	1.96	1.64	41.6
212	Rice, boiled	Basmati	0.07	0.02	1.37
213	Rice, fried	Boiled, then fried	0.96	1.32	8.59
214	Rice, fried	Fried in olive oil	0.12	0.03	4.17
215	Pasta, boiled	Penne	0.59	0.11	2.51
216	Pasta, boiled	Spaghetti	0.82	0.13	3.02
217	Pasta, boiled	Macaroni	0.43	0.06	1.41

**Data expressed as mean±sd



DIETARY INTAKE OF *advanced glycation endproducts* is associated with higher levels of advanced glycation endproducts in plasma and urine: THE CODAM STUDY

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Dietary intake of advanced glycation endproducts is associated with higher levels of advanced glycation endproducts in plasma and urine: the CODAM study

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Abstract

Background & Aims: Advanced glycation endproducts (AGEs) are formed by the reaction between reducing sugars and proteins. AGEs in the body have been associated with several age-related diseases. High-heat treated and most processed foods are rich in AGEs. The aim of our study was to investigate whether dietary AGEs, are associated with plasma and urinary AGE levels.

Methods: In 450 participants of the Cohort on Diabetes and Atherosclerosis Maastricht study (CODAM study) we measured plasma and urine concentrations of the 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) using UPLC-MS/MS. We also estimated dietary intake of CML, CEL and MG-H1 with the use of a dietary AGE database and a food frequency questionnaire (FFQ). We used linear regression to investigate the association between standardized dietary AGE intake and standardized plasma or urinary AGE levels, after adjustment for age, sex, glucose metabolism status, waist circumference, kidney function, energy- and macronutrient intake, smoking status, physical activity, alcohol intake, LDL-cholesterol and markers of oxidative stress.

Results: We found that higher intake of dietary CML, CEL and MG-H1 was associated with significantly higher levels of free plasma and urinary CML, CEL and MG-H1 (βCML=0.253 (95% CI 0.086; 0.415), βCEL=0.194 (95% CI 0.040; 0.339), βMG-H1=0.223 (95% CI 0.069; 0.373) for plasma and βCML=0.223 (95% CI 0.049; 0.393), βCEL=0.180(95% CI 0.019; 0.332), βMG-H1=0.196 (95% CI 0.037; 0.349) for urine, respectively). In addition, we observed non-significant associations of dietary AGEs with their corresponding protein bound plasma AGEs.

Conclusion: We demonstrate that higher intake of dietary AGEs is associated with higher levels of AGEs in plasma and urine. Our findings may have important implications for those who ingest a diet rich in AGEs.

Introduction

Advanced glycation endproducts (AGEs) are a large and heterogeneous family of sugar-modified proteins, which have been linked to a plethora of age-related diseases including diabetes, atherosclerosis, chronic obstructive pulmonary disease and Alzheimer's disease[1-3].

In 1912, the French chemist Louis Camile Maillard discovered the reaction between reducing sugars and free amino acids on proteins[4], leading to the formation of AGEs. At body temperature, the Maillard reaction takes place at a very slow pace. In contrast, in foods which had a prolonged exposure to high heat AGEs were formed rapidly[5]. The consumption of processed foods that were cooked at high temperatures has increased over the past decades[6]. As a consequence, the exposure to dietary AGEs have also increased and may be a risk factor for chronic diseases[7]. Indeed, recent studies have shown associations of dietary AGEs with insulin sensitivity[8], abdominal obesity and hypertriglyceridemia[9] and with poorer memory in Alzheimer's disease[10]. AGEs in the body may contribute to development of age-related diseases through several mechanisms, such as interaction with the receptor for AGEs (RAGE)[11] and crosslinking on long-lived proteins[12]. However, whether dietary AGEs are substantially absorbed in the digestive tract and released into the circulation to contribute to any of these effects remains unclear.

The studies so far addressing whether high intake of dietary AGEs lead to increased plasma and urinary AGEs have not been conclusive[13-22], because most of these studies have been small and have not taken into account confounding factors such as kidney function or dietary energy intake, which may explain the associations[19,20]. Moreover, different analytical techniques to analyze AGE content in food and plasma samples were used in these studies, which may have led to differences in AGE concentrations[21,22].

Taken these considerations into account, the aim of the current study was to establish whether higher intake of dietary AGEs are associated with higher levels of plasma and urinary AGEs. In a previous study, we developed a new dietary AGE database of 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), as analyzed in 190 specific food items, based on a state-of-the art ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) technique[23]. In the present study, we linked information on AGE-content of these food items to a food frequency questionnaire (FFQ) and calculated the consumption of AGEs in a cohort of 450 participants. We subsequently examined the association of dietary AGE intake with three major plasma and urinary AGEs (CML CEL and MG-H1), adjusting for major potential confounders.

Materials and methods

Study population

Cross-sectional analyses were performed on data from the Cohort on Diabetes and Atherosclerosis Maastricht study (CODAM), which includes 574 individuals with an elevated risk for T2DM and cardiovascular disease and described in detail elsewhere[24]. Participants were instructed to withhold their lipid-lowering medication for a fortnight prior the first visit, and not to consume any alcoholic drinks, coffee and/or tea, not to smoke, and withhold all other medication the day before. The habitual dietary intake over the last twelve months of all participants was established by a self-administered food frequency questionnaire (FFQ) which queried 194 foods[25].
Individuals were excluded if they did not qualify to report the FFQ successfully (n=56, i.e. more than 10% items missing on the FFQ). Participants who reported an energy intake outside the range of 800-4200 kcal/day for men and 600-3500 kcal/day for women were also excluded (n=6). Due to sample availability, 450 participants were used for statistical analysis (figure 9.1).
Fasting and 2-h postload glucose concentrations were used to classify the study participants' glucose metabolism status (GMS), described in details elsewhere[26]. Questionnaires were used to assess smoking behavior (never, ever, or current smoker) and use of medication (lipid-, glucose-, and blood pressure-lowering medication). Plasma creatinine levels were measured with the Jaffe diagnostic test (Roche Diagnostics, Mannheim, Germany), and the estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation[27]. Plasma malondialdehyde (MDA) levels were measured with a reagent kit for high-performance liquid chromatography analyses (Chromsystems Instruments and Chemicals, Munich, Germany), and total antioxidative status (TAS) was measured in serum with an enzymatic kit (Randox Diagnostics, County Antrim, U.K.). LDL cholesterol was calculated with the Friedewald formula[28], after measurement of fasting total cholesterol, HDL-cholesterol and triglyceride levels.
All subjects gave written informed consent. The study was approved by the local Medical Ethical Committee of the University of Maastricht and University Hospital Maastricht.

Food, plasma and urinary AGE quantification

Protein-bound AGEs in individual food items and in plasma, and free AGEs in plasma and in urine were analyzed as described in detail elsewhere[23,29]. In short, foods were obtained from local supermarkets or restaurants and prepared according to the instructions on the label. For UPLC-MS/MS analysis a representative sample of each food item, equivalent to 5 mg of protein, was used. For protein-bound and free plasma AGEs, 25 µL and 50 µL plasma was used, respectively.

Forty microliters were used for free urinary AGE analysis. 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 CML, CEL and 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%. In food matrix, the intra- and inter-assay variation of protein-bound CML, CEL and MG-H1 were between 1.7 and 29.1%.

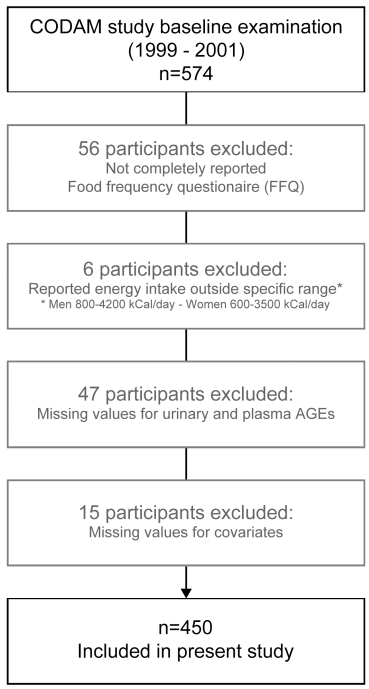

Figure 9.1 Flowchart for the exclusion of participants in the current CODAM study.

Assessment of AGE intake

For UPLC-MS/MS protein-bound AGE analysis, food items were selected based on the items included in the FFQ of CODAM, which queried 194 foods commonly consumed in the Netherlands. Each food item in the FFQ was quantified as grams of food per day and analyzed for AGE content. The AGE intake was estimated for each participant by multiplying the food intake in g/day with the concentration of AGEs in mg/g in each representative individual food item.

To calculate the total AGE intake per participant, the estimated AGE levels of the various food items were added together. Some of the food items had unspecific descriptions in the FFQ (e.g. "sweet spreads"). These items were further defined as an average of this food group (e.g. for "sweet spreads" the average AGE content of apple syrup, chocolate spread, chocolate sprinkles, fruit sprinkles and jam was used). Moreover, some of the food items in the FFQ were not analyzed for AGEs. These food items were matched with food items based on ingredient profile. To this end, estimation was made for the AGE content of these missing food items.

Statistical analyses

All statistical analyses were carried out using the IBM SPSS Statistics Software, version 23.0 (IBM Corporation, Armonk, NY, USA). General characteristics of the CODAM population were compared across tertiles of the dietary AGE score with the use of ANOVA or X^2-tests, for continuous or categorical data, respectively. We $\log_e$-normalised all plasma and urinary AGEs to reduce the potential influence of outliers and to obtain a normal distribution, needed for calculation of Z-scores. An AGE score was calculated that represents an individual's overall intake of AGEs via their habitual diet. For this, first the Z-scores were calculated for each dietary AGE (CML, CEL and MG-H1) as follows: for each individual dietary AGE, a Z-score was calculated according to the formula: [(individual dietary AGE –population mean dietary AGE)/population standard deviation dietary AGE]. The resulting Z-scores, of each dietary AGE (CML, CEL and MG-H1), were then averaged into an overall dietary AGE Z-score and this overall dietary AGE Z-score was subsequently standardized.

We used linear regression analyses to investigate the association between dietary and plasma or urinary AGE levels, after adjustments for participant characteristics (age, sex, glucose metabolism status and waist circumference: model 1) and additionally for kidney function (estimated glomerular filtration rate (eGFR): model 2), and further for potential dietary and lifestyle confounding factors (energy intake and protein-, saturated fat-, carbohydrate- and fibre-intake, all macronutrients expressed as energy percentage and smoking, physical activity and alcohol intake: model 3), and for potential confounding by lipid metabolism and oxidative stress (LDL-cholesterol, malondialdehyde (MDA) and total antioxidant status (TAS): model 4). Participants with missing values (n=14) on co-variates were excluded listwise. We also tested whether associations differed for sex (sex x dietary AGE), eGFR (eGFR x dietary AGE) and the presence of impaired glucose metabolism or diabetes (presence of impaired glucose metabolism or diabetes x dietary AGE), by adding interaction terms to our models (p-values for interaction were <0.1). Overall, we found no consistent interactions (data not shown).

Results

Table 9.1 shows the general characteristics of the total population of the CODAM study as stratified according to tertiles of dietary AGEs. Although we observed important quantitative differences in dietary AGE levels, with MG-H1 being most abundant, we observed strong correlations between the AGE intake (CML vs. CEL $r_{pearson}$: 0.811, CML vs. MG-H1 $r_{pearson}$: 0.795 and CEL vs. MG-H1 $r_{pearson}$: 0.880; for all $p<0.001$). Plasma free-, plasma protein-bound- and urinary AGEs did not differ between the tertiles of dietary AGEs (table 9.1). Male participants had higher intake of dietary AGEs ($p<0.001$, table 9.1). In addition, HDL cholesterol, protein intake and fibre intake were lower in the individuals with higher intake of dietary AGEs ($p<0.05$, table 9.1) and saturated fat-, energy- and alcohol intake were higher in the individuals with higher intake of dietary AGEs ($p<0.05$, table 9.1).

Associations between dietary AGE intake and protein-bound plasma AGEs

After adjustment for age, sex, glucose metabolism status and waist circumference, we found no significant association between dietary CML and protein-bound plasma CML levels (table 9.2, model 1). Further adjustment for eGFR did not change these results (table 9.2, model 2). In the fully adjusted model we observed a weak positive association of dietary CML and protein-bound plasma CML, but this was not significant (table 9.2, model 4). We observed similar findings for dietary CEL and protein-bound CEL (table 9.2).

Associations between dietary AGE intake and free plasma AGEs

After adjustment for age, sex, glucose metabolism status and waist circumference we observed positive associations between dietary intake of CML, CEL and MG-H1 and their free levels in plasma (table 9.3, model 1). Further adjustment for eGFR (table 9.3, model 2), energy- and macronutrient intake, lifestyle factors, lipid metabolism and markers of oxidative stress (table 9.3, model 3 and 4) did not change the results overall. In the fully adjusted model, these associations were approximately twice as strong for CML, CEL and MG-H1 (table 9.3, model 4 vs. model 1).

Associations between dietary AGE intake and free urinary AGEs

In line with the findings for free plasma AGEs, higher dietary intake of CML, CEL and MG-H1 were associated with higher urinary CML, CEL and MG-H1 levels (table 9.4, model 1-4). Adjustment for eGFR did not change the results (table 9.4, model 2).

Table 9.1 Clinical characteristics of total study population

	Total population	Dietary AGE tertiles			
	(n=450)	Lowest (n=151)	Middle (n=151)	Highest (n=148)	P_{trend}
Age (years)	59.4 ± 7.0	60.1±6.9	59.0±6.7	58.9±7.4	0.140
Sex (m/f; %)	60.0/40.0	45.7/54.3	59.6/40.4	75.0/25.0	<0.001
NGM/IGM/T2DM (%)	53.6/22.7/23.8	50.3/23.2/26.5	56.3/18.5/25.2	54.1/26.4/19.6	0.376
Body mass index (kg/m^2)	28.3 ± 4.2	28.5±4.5	28.2±4.1	28.3±3.9	0.690
Waist circumference (cm)	98.6 ± 11.6	97.8±12.1	98.4±11.7	99.6±11.0	0.162
Fasting glucose (mmol/L)	6.0 ± 1.4	6.0±1.3	6.0±1.5	6.0±1.4	0.823
HbA1c (%)	5.9 ± 0.8	5.9±0.6	6.0±0.9	6.0±0.8	0.671
Total cholesterol (mmol/L)	5.2 ± 0.9	5.3±0.9	5.1±1.0	5.2±0.8	0.553
HDL cholesterol (mmol/L)	1.20 ± 0.35	1.25±0.36	1.22±0.35	1.15±0.33	0.010
LDL cholesterol (mmol/L)	3.29 ± 0.86	3.32±0.90	3.21±0.89	3.35±0.79	0.757
Triglycerides (mmol/L)	1.4 (1.0-1.9)	1.4 (1.0-1.9)	1.4 (1.0-2.0)	1.4 (1.0-1.9)	0.969
Current smoking (%)	20.2	21.1	22.3	18.5	0.756
Malondialdehyde (µmol/L)	0.18 ± 0.05	0.18±0.06	0.17±0.05	0.18±0.05	0.887
Total antioxidant status (mmol/L)	1.08 ± 0.10	1.08±0.10	1.07±0.10	1.10±0.10	0.090
SBP (mm Hg)	139.3 ± 18.7	141.4±17.9	138.4±19.9	138.2±18.0	0.144
DBP (mm Hg)	81.3 ± 9.0	81.6±8.2	80.4±9.5	82.0±9.3	0.697
eGFR (mL/min/1.73 m^2)	91.8 ± 17.3	93.1±23.9	90.8±12.6	91.4±12.7	0.379
Glucose-lowering treatment (%)	13.3	13.2	14.6	12.2	0.828
Lipid-lowering treatment (%)	19.1	19.9	21.2	16.2	0.527
Blood pressure-lowering treatment (%)	38.0	41.7	39.1	33.1	0.292
Energy intake (kcal/day)	2191.9 ± 601.0	1677.9 ± 382.0	2148.2 ± 351.6	2750.8 ± 478.5	<0.001
Protein intake (% energy)	15.5 ± 2.2	15.9 ± 2.5	15.6 ± 2.1	15.1 ± 1.9	0.002
Carbohydrate intake (% energy)	44.0 ± 6.2	43.8 ± 6.9	44.4 ± 5.4	43.9 ± 6.2	0.943
Saturated fat intake (% energy)	14.6 ± 2.5	14.2 ± 2.6	14.6 ± 2.5	15.0 ± 2.5	0.006
Fibre intake (% energy)	2.36 ± 0.49	2.47 ± 0.51	2.34 ± 0.49	2.25 ± 0.45	<0.001
Alcoholic drinks (g/day)	107 (11-280)	71 (6-225)	100 (12-284)	135 (29-304)	0.032
Physical activity (min*intensity/week)	1800 (1058-2835)	1703 (1043-2655)	1890 (1140-2865)	1837 (956-2966)	0.404
Plasma free CML (nmol/L)	78 (60-99)	77 (57-101)	77 (63-99)	80 (60-97)	0.626
Plasma free CEL (nmol/L)	45 (37-58)	45 (35-59)	44 (38-55)	47 (38-60)	0.496
Plasma free MG-H1 (nmol/L)	121(86-173)	113 (82-167)	120 (91-166)	126 (88-191)	0.117
Plasma protein-bound CML (nmol/mmol lys)	35 (30-41)	35 (29-41)	34 (31-41)	35 (29-42)	0.861
Plasma protein-bound CEL (nmol/mmol lys)	24 (19-29)	22 (18-27)	24 (19-29)	24 (20-30)	0.056
Urinary free CML (nmol/mmol creatinine)	954 (749-1220)	944 (735-1257)	941 (739-1244)	966 (756-1175)	0.980
Urinary free CEL (nmol/mmol creatinine)	519 (409-645)	519 (428-635)	510 (384-631)	540 (405-675)	0.296
Urinary free MG-H1 (nmol/mmol creatinine)	2284 (1593-3270)	2251 (1587-3286)	2274 (1535-3190)	2421 (1664-3397)	0.627
Dietary CML (mg/day)	3.10 ± 1.03	2.08±0.47	3.07±0.44	4.17±0.77	<0.001
Dietary CEL (mg/day)	2.32 ± 0.77	1.58±0.31	2.24±0.28	3.15±0.60	<0.001
Dietary MG-H1 (mg/day)	21.7 ± 6.69	15.2±2.85	20.9±2.42	28.9±5.09	<0.001

Data are presented as means ± SD, medians (IQR) or percentages, as appropriate. Linear trend was tested with ANOVA or $\chi 2$, as appropriate.

Skewed continuous variables were analyzed with Kruskal Wallis (triglycerides, and the AGEs) were loge-transformed prior to analyses.

Table 9.2 Associations between dietary AGEs and protein-bound plasma AGEs.

	Protein-bound plasma CML			
	Model	β	95%CI	*p*
Dietary CML	Crude	0.039	-0.054; 0.128	0.42
	1	0.048	-0.041; 0.133	0.30
	2	0.046	-0.042; 0.131	0.32
	3	0.122	-0.042; 0.277	0.15
	4	0.130	-0.032; 0.283	0.12

	Protein-bound plasma CEL			
	Model	β	95%CI	*p*
Dietary CEL	Crude	0.112	0.017; 0.199	0.02
	1	0.105	0.005; 0.197	0.04
	2	0.101	0.002; 0.194	0.05
	3	0.136	-0.030; 0.293	0.11
	4	0.112	-0.050; 0.268	0.18

Table 9.3 Associations between dietary AGEs and free plasma AGEs.

	Free plasma CML			
	Model	β	95%CI	*p*
Dietary CML	Crude	0.055	-0.039; 0.147	0.26
	1	0.099	0.006; 0.190	0.04
	2	0.094	0.004; 0.183	0.04
	3	0.224	0.057; 0.386	0.01
	4	0.253	0.086; 0.415	<0.01

	Free plasma CEL			
	Model	β	95%CI	*p*
Dietary CEL	Crude	0.086	-0.008; 0.176	0.07
	1	0.098	0.003; 0.188	0.04
	2	0.086	-0.007; 0.174	0.07
	3	0.208	0.053; 0.353	0.01
	4	0.194	0.040; 0.339	0.01

	Free plasma MG-H1			
	Model	β	95%CI	*p*
Dietary MG-H1	Crude	0.127	0.033; 0.219	0.01
	1	0.150	0.056; 0.241	<0.01
	2	0.135	0.043; 0.224	<0.01
	3	0.239	0.085; 0.390	<0.01
	4	0.223	0.069; 0.373	<0.01

Table 9.4 Associations between dietary AGEs and free urinary AGEs.

	Free urine CML			
	Model	β	95%CI	*p*
Dietary CML	Crude	0.045	-0.049; 0.138	0.35
	1	0.127	0.033; 0.218	0.01
	2	0.127	0.033; 0.218	0.01
	3	0.211	0.038; 0.380	0.02
	4	0.223	0.049; 0.393	0.01

	Free urine CEL			
	Model	β	95%CI	*p*
Dietary CEL	Crude	0.056	-0.037; 0.146	0.25
	1	0.121	0.024; 0.211	0.01
	2	0.124	0.027; 0.214	0.01
	3	0.189	0.027; 0.340	0.02
	4	0.180	0.019; 0.332	0.03

	Free urine MG-H1			
	Model	β	95%CI	*p*
Dietary MG-H1	Crude	0.066	-0.028; 0.157	0.17
	1	0.134	0.039; 0.224	<0.01
	2	0.134	0.043; 0.228	<0.01
	3	0.205	0.045; 0.357	0.01
	4	0.196	0.037; 0.349	0.02

Table 9.2, 9.3 and 9.4:
Data were analyzed using linear regression analyses. βs are expressed as 1 SD increase of urinary/plasma AGE per 1SD increase of dietary AGE. All plasma and urinary AGEs were Ln-normalised in all analyses.
Model 1: adjusted for age, sex, glucose metabolism status, waist circumference
Model 2: model 1 + eGFR
Model 3: model 2 + total energy-, protein-, saturated fat-, carbohydrate- and fibre-intake, smoking, physical activity, alcohol intake
Model 4: model 3 + LDL cholesterol, malondialdehyde, total antioxidant status

Discussion

This study is, to our knowledge, the first to demonstrate that higher dietary intake of CML, CEL and MG-H1, as estimated with a FFQ, is associated significantly with higher free plasma and urinary levels of AGEs, as measured with UPLC-MS/MS. We found a weak non-significant association of dietary AGEs with their corresponding protein bound plasma AGEs. In the current study we estimated dietary CML, CEL and MG-H1. Since these compounds were highly correlated, despite quantitative differences, we assume that these three AGEs were well-representative for dietary AGE contents.

Studies so far have suggested that dietary AGEs are associated with circulating AGEs[13,15-18,30]. Our current study expands on these findings in several important ways. First, we have used a state-of-the-art UPLC-MS/MS technique to analyze three different dietary AGEs and circulating AGEs. Moreover, we performed UPLC-MS/MS plasma and urinary analyses in a large and well-defined cohort study to investigate the associations between three different dietary AGEs and plasma and urinary AGEs. Another major strength of our study is that we were able to use a database of AGEs, as measured with UPLC-MS/MS[23], in 190 food items to calculate the habitual intake of dietary AGEs based on a FFQ. However, one important limitation of our study was that our FFQ did not include detailed information about food preparation techniques on all food items. We and others have shown that differences in these cooking techniques and duration of heating are fundamental in the formation of AGEs in food[5,23,31]. However, approximately 90% of the FFQ food items were either uncooked, such as fruit, raw vegetables and drinks or were pre-processed foods which were produced by different manufacturers, such as cookies, bread or peanut butter. Our FFQ consisted of only 10% of food items which have to be prepared by the participant itself, such as meat, fish, potatoes or pasta. We have included detailed information on the preparation of potatoes in the FFQ, such as boiling, frying or baking. Unfortunately, no detailed information of the preparation, in particular duration of heating, of meat or fish was available. However, our dAGE database[23], which was used to estimate AGE-intake as calculated with FFQ data, consisted of meat- and fish products which were prepared according to the instructions of the manufacturer's label or using the most common preparation technique. Perhaps this will have led to a source of variance in estimating the dietary AGE content, but this was only true for a minority of the food items. Nonetheless, we still found clear and consistent associations between estimated dietary AGE intake and plasma AGE levels.

Our crude analysis only revealed an association between higher dietary AGEs and higher plasma and urinary AGE levels. Particularly after adjustment for sex, age, glucose metabolism and waist (model 1), and further adjustment for the major macronutrients and lifestyle factors (model 3) even stronger associations were found between dietary AGEs and free plasma and urinary AGEs. The strong effect of confounding factors on the reported associations may at least partly explain conflicting findings between previous studies which have not taken into account these confounding factors[19,20].

Dietary AGE-modified proteins are mainly digested into free glycated and non-glycated amino acids and small peptides[32] and thus protein-bound dietary AGEs are expected to enter the circulation predominately in the free form. Subsequently, these AGEs are excreted in the urine[16]. We found no association of dietary AGEs with protein-bound AGEs in plasma, suggesting that the vast majority of the protein-bound AGEs in the human body are formed by the non-enzymatic reaction of glucose, α-oxoaldehydes and other saccharide derivatives with proteins, nucleotides and lipids. On the contrary, we found an association of dietary AGEs with free AGEs in plasma. This is in accordance with a recent study which has showed that free plasma AGEs were mainly released from the protein-bound dietary AGEs and hereby enter the circulation predominantly[32]. In line, we found that associations between dietary AGEs and free plasma AGEs are stronger than

with protein-bound plasma AGEs. Two intervention studies were in line with these results[13,16]. Birlouez-Aragon et al, reported a significant increase of 7% protein-bound plasma CML after administering a high AGE diet versus a significant increase of 40% free urinary CML[16]. Furthermore, the associations between the estimated dietary AGE intake with free plasma AGEs is in line with animal intervention studies[33,34]. In these studies, low- and high-AGE diets were tested in animals and significantly increased levels of free plasma CML, but not protein-bound plasma CML, were found[33,34]. In the same study, increased plasma levels of sRAGE and a pro-inflammatory response was reported after administering a high-AGE diet[33] which implicates important biological consequences.
In humans, the beneficial effects of a low AGE-diet have been described[8,13,35]. If a high-AGE diet in humans, and contemporaneously increased levels of free plasma AGEs, can lead to an increased risk for development of chronic diseases is still under investigation. In our observational study we investigated the association of a habitual diet with similar results.
In summary, we have shown that higher levels of dietary AGEs were significantly associated with higher levels of free plasma and urinary AGEs. These findings are an important step in understanding the metabolic transit of these dietary AGEs. Several studies indicate that dietary AGEs are associated with poor health[8-10,16,20]. Future prospective studies should address whether dietary AGEs are associated with adverse outcomes such as accelerated development of cardiovascular disease, diabetes and other age-related diseases. This line of investigation may eventually lead to new dietary recommendations for both the general public and perhaps specific patients groups in particular.

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10 Summary & GENERAL discussion

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Diabetes mellitus is a chronic, metabolic disease characterized by elevated levels of blood glucose[1], and is an established risk factor for coronary heart disease and ischaemic stroke[2,3]. It is affecting approximately 422 million people worldwide and has become a major public health concern[4,5]. Hyperglycaemia leads to reactive dicarbonyl compounds such as methylglyoxal[6] and increased formation of advanced glycation endproducts (AGEs). To investigate the consequences of dicarbonyl compounds and AGEs for human health, sensitive and reliable analytical techniques are needed. The aim of the research described in this thesis was to develop analytical techniques to measure dicarbonyl compounds and AGEs and to apply these techniques in clinical studies.

Advanced glycation endproducts; development of analytical techniques and its application

In the early beginning of AGE research, AGEs were detected and quantified as a group of AGEs with fluorescent properties[7,8]. However, quantification of specific fluorescent AGEs such as crossline[9], pentosidine[10], vesperlysine A, B and C[11], MRX[12] and pyrrolopyridinium[11], was not possible with this technique. Later on, specific AGEs were quantified by enzyme-linked immunosorbent assays (ELISA)[7,13-17] but specificity, reproducibility and sensitivity of this technique were not optimal[18-20]. A better approach for the quantitative determination of AGEs is the use of a specific analytical technique.

In chapter 2A, a rapid and sensitive single-column high performance liquid chromatography (HPLC) method and application for the detection of protein bound pentosidine is described. Many techniques were used to quantify pentosidine[21-27] with some disadvantages such as long run-times[21,24,28], elaborate sample preparation[29] or analysis on expensive equipment[25,30]. The described technique in chapter 2A has overcome these shortcomings and presented a very clean chromatogram with a very stable retention-time and a run-to-run time of only 30 minutes. Moreover, the sample preparation is also suitable for the simultaneous analysis of protein-bound N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) and lysine (chapter 2B). With this improved technique protein-bound pentosidine was analyzed in plasma samples of healthy volunteers and patients on peritoneal dialysis (PD). A 15-fold increase of plasma protein-bound pentosidine was found in uremic patients when compared to healthy controls.

In chapter 2B a sensitive, simple and highly selective method to determine protein-bound and free CML, CEL and MG-H1 in plasma by ultra-performance liquid chromatography (UPLC-MS/MS) is described.
Many techniques including ELISA[7,13,15-17], HPLC[31-34], gas chromatography mass spectrometry (GC-MS)[18,35] and liquid chromatography mass spectrometry (LC-MS)[17,25,34,36], have been described to determine AGEs in plasma and other matrices. However, all these techniques have several limitations. For the ELISA, standardization is difficult, immunoreactivity of the antibody and AGE epitope can be affected by numerous factors and sensitivity and specificity is often a matter of concern[15]. HPLC and LC-MS uses elaborate and expensive enzymatic hydrolysis[25,32,34] which is not desirable for large cohort studies. Moreover, several analytical methods analyzed just one or two AGEs[17,18,36]. The described method in chapter 2B has overcome these limitations and presented a well validated, sensitive, simple and highly selective method to determine CML, CEL and MG-H1 in plasma. With this technique, protein-bound and free plasma AGEs were analyzed in healthy volunteers and patients on PD. Increased plasma levels of protein-bound and free AGEs were found to be increased in patients on PD as compared to healthy controls. This was in reasonable agreement with data described in literature[36-41]. The described method is suitable for studying AGEs in several different human and animal studies[42-49] and is a strong tool to investigate the putative effects of AGEs in the pathophysiology of different diseases.

In chapter 3, the analytical methods as described in chapter 2A and B, were used to investigate the associations of plasma AGEs with cardiovascular disease (CVD) in individuals with or without type 2 diabetes mellitus (T2DM). In this cross-sectional analysis of a combined sample of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) and the Hoorn study, we found no associations of plasma protein-bound and free AGEs with glucose metabolism status and prevalent CVD. These results might be somewhat confusing since the putative effects of AGE accumulation on risk of CVD have been described previously[50,51] and higher plasma AGE levels were found in type 1 diabetes mellitus (T1DM)[52]. However, given the cross-sectional design of the presented study, we cannot completely rule out the lack of associations of plasma AGEs with prevalent CVD. Possible lifestyle interventions, such as glucose-, blood-pressure- and lipid-lowering treatments[50,53,54] and changes in dietary AGEs (due to diet interventions), may potentially influence circulating AGEs. Moreover, CML has been found to be trapped in adipose tissues[42,55] and consequently, plasma CML levels are lower in individuals with overweight compared to lean individuals[55]. Given the high prevalence of overweight and obesity in patients with T2DM, this phenomenon manifests more often in T2DM as compared to T1DM or lean individuals.

D-lactate as a reflection of methylglyoxal?

Methylglyoxal (MGO), a highly reactive dicarbonyl compound generated by the spontaneous degradation of glycolytic intermediates, is a major precursor for AGEs and can potentially disrupt cellular functions. To investigate the glyoxalase pathway, in particular the detoxification of this MGO into D-lactate, there is need of a sensitive and highly specific method to determine D-lactate. Several different techniques have been described with some shortcomings such as low sensitivity[56-58], large sample volume[59-61], complex chromatographic systems[62-64] and long run times[56,58,62,65]. In chapter 4 a highly sensitive, specific and fast UPLC-MS/MS method to determine L- and D-lactate in plasma and urine is described. Without the need of cumbersome chiral stationary phases or complex chromatographic systems, a significant improvement over the methods described in literature was achieved. Because the origin of D-lactate may be derived from the production of intestinal bacteria and degradation of methylglyoxal by the glyoxalase pathway L- and D-lactate was determined in plasma and urine samples of controls, patients with inflammatory bowel disease (IBD) and patients with T2DM. Increased plasma and urinary levels of L- and D-lactate in patients with IBD and T2DM compared to controls were found, but only significantly increased in patients with T2DM. Although these findings strongly suggest that the increased levels of D-lactate in patients with T2DM is a reflection of MGO, we cannot definitely exclude the possibility that D-lactate is a reflection of gut flora, or gut permeability.

In chapter 5, we have further explored the association between plasma D-lactate and insulin resistance. In this study the association of plasma D-lactate, as a reflection of MGO levels, and insulin resistance (HOMA2-IR) was investigated by multiple linear regression analysis. A positive association between plasma D-lactate and HOMA2-IR was found, independently of potential confounders. Moreover, an increase of plasma D-lactate levels in patients with T2DM was demonstrated. These results are in agreement with the results described in chapter 4 and suggest that MGO plays a role in insulin resistance. However, the question remained if D-lactate is a reflection of MGO levels. For answering this question, a direct measurement of MGO was needed.

α-Oxoaldehydes and type 2 diabetes

Numerous techniques have been described for the quantification of the reactive α-oxoaldehydes glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) including reversed phase liquid chromatography with fluorescence or UV detection[66-71] or liquid chromatography with mass spectrometry detection[72-76]. However, these techniques have several shortcomings such as large sample volume[67,69,72,73], elaborate liquid-liquid extraction (LLE) or solid phase extraction (SPE) steps[66,67,72-75] and long run-times[66,68,71].
In chapter 6 a highly sensitive, specific and rapid ultra-performance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) method for the analysis of

GO, MGO and 3-DG is described. A significant improvement over the methods described in the literature was achieved with the use of a stable isotope labeled internal standard. Sample preparation techniques, variations in sample treatment and storage and also the interval between blood draw and sample analysis can influence the free concentrations of α-oxoaldehydes. Standardization of these conditions is necessary to obtain reliable and reproducible results.
Therefore, blood has to be withdrawn in EDTA tubes and subsequently derivatized as plasma with O-phenylenediamine (oPD) in the presence of perchloric acid (PCA). In particular, EDTA tubes with added sodium fluoride (NaF) should be avoided because inhibition of the enolase by NaF[77] may lead to the accumulation of precursors of MGO and GO.
With this improved technique, increased plasma concentrations of GO, MGO and 3-DG were found in patients with T2DM as compared to healthy individuals. These results were in reasonable agreement with data described in literature[69,78-80]. Nevertheless, absolute concentrations differ between studies and are most likely due to differences in sample preparation and sample handling during storage. Moreover, these results support that D-lactate is most likely a reflection of the detoxification of MGO by the glyoxalase system as stated in chapter 4 and 5.

Patients with diabetes or impaired glucose metabolism are at higher risk for the development of micro- and macrovascular complications and CVD[81]. As a consequence of a disturbed glucose metabolism, glucose-derived reactive α-oxoaldehydes are formed (chapter 6) and may lead to diabetic complications[78,79,82,83]. However, it is unknown to what extent the circulating α-oxoaldehydes are affected after a postprandial glucose excursion. Postprandial glucose excursions have increasingly been recognized as a contributing factor to the increased risk of vascular complications[84,85].
In chapter 7, we have investigated whether α-oxoaldehydes are increased after a glucose load in individuals with normal and impaired glucose metabolism (NGM and IGM) and in patients with T2DM. Plasma α-oxoaldehydes were determined in the fasting state and after an oral glucose tolerance test (OGTT), by UPLC-MS/MS (method described in chapter 6). Cross-sectional, linear analysis was performed in the CODAM study, consisting of 574 individuals.
This study demonstrated a positive and significant association of glucose metabolism status with higher α-oxoaldehyde incremental area under the curve (iAUC), as calculated from an OGTT. This association was independent of potential confounders. Moreover, fasting plasma levels of MGO and 3-DG were predominantly higher in patients with T2DM. However, after adjustment for glucose, these associations disappeared completely, indicating glucose as a major source for these α-oxoaldehydes.

These results are in agreement with data described by Beisswenger et al.[86], who found increased plasma levels of MGO and 3-DG in patients with T1DM after a postprandial glycemic excursion.
Since higher levels of α-oxoaldehydes are directly associated with vascular complications[44,87] and formed directly from glucose, postprandial periods may lead to an increased risk of vascular dysfunction. Consequently, fluctuations in blood glucose levels, in particular in patients with IGM or diabetes, may lead to increased α-oxoaldehyde levels and thus increases risk for vascular complications and CVD. These findings underline the potential importance of dicarbonyl stress as a functional candidate to explain the increased risk of diabetic complications in individuals with postprandial hyperglycemia.

Advanced glycation endproducts in foods

Accumulation of AGEs in the body has been implicated in the pathogenesis of age-related diseases in particular in relation to diabetes, inflammation, neurodegenerative disorders and cardiovascular disease[46,88-90]. Since the processing and preparation of foods is also susceptible to AGE formation with high temperatures as a potent promoter[91-94] dietary AGEs may influence the endogenous AGE pool. Animal studies have shown that some of these dietary AGEs are absorbed and have pathological effects, such as inducing insulin resistance[92,95,96]. In humans, dietary AGE intervention studies have shown to increase circulating AGEs and markers of inflammation and endothelial dysfunction, and to impair flow-mediated dilation and insulin sensitivity[97-101]. Nevertheless, many studies on dietary AGEs are based on semi-quantitative techniques, such as ELISA, and should be interpreted with care[18,92]. Therefore, a sensitive and reliable analytical method to determine the AGE content in foods was needed.

In chapter 8, a UPLC-MS/MS method for the determination of CML, CEL and MG-H1 in foods was validated. A database of AGE content in 190 food items, commonly consumed in a western diet, was presented. Overall, the food items which were heated, had long processing times, and were industrially conserved or products containing chocolate, nuts or grains were high in AGE content. Food items with a short heat processing time, low protein and/or carbohydrate content or high water content had low or negligible levels of AGEs. For CML, these data were in line with data from Hull et al.[102] who presented a database of CML content in 257 food items as analyzed with the same technique. However, absolute concentrations differ, most likely due to differences in preparation techniques[91,94,102-107]. Three other major studies were described, but these studies were based on ELISA techniques[107-109] and show inconsistent results. Since ELISA based techniques are sensitive for interference of the lipid matrix[18] and steric hindrance of the antigen-antibody interaction as a consequence of matrix interferences[92] these data should be interpreted with care.
The main advantages of the study, presented in chapter 8, are the use of a state-of-the-art UPLC–tandem MS technique and the ability to analyze three major AGEs in one run.

The presented AGE database and the validated analytical method are strong tools to further investigate the relevance of dietary AGEs in disease.
Intervention studies, with low and high AGE diets, to study the health effect of AGEs in much more detail are now possible.
However, whether dietary AGEs are substantially absorbed in the digestive tract and released into the circulation to contribute to any of these effects remains unclear. So far, studies investigating whether higher intake of AGEs is associated with circulating AGEs showed inconsistent results[19,97-100,110-116], because most of these studies have been small, have not taken into account confounding factors or used different techniques to analyze AGEs.

In chapter 9, these considerations have been taken into account and the associations of dietary CML, CEL and MG-H1 with plasma and urinary levels of their corresponding AGEs were investigated. In 450 participants of the CODAM study, plasma and urinary levels of CML, CEL and MG-H1 were determined, using the method described in chapter 2B. Dietary intake of CML, CEL and MG-H1 was estimated with the dietary database (chapter 8) and a food frequency questionnaire (FFQ). A positive and statistically significant association was found between dietary CML, CEL and MG-H1 and their corresponding free plasma and urinary levels. In addition, a positive but non-significant association was found between dietary AGEs and their corresponding protein bound plasma AGEs. These results are in line with previous studies[97,98,100,111-113], but the current study expands on these findings in several important ways. The presented study used state-of-the-art UPLC-MS/MS technique to analyze three different dietary AGEs and circulating AGEs. Statistical analysis was done in a large and well-defined cohort study to investigate the associations between dietary AGEs and plasma and urinary AGEs corrected for potential confounders. Moreover, a reliable database of AGEs (chapter 8) was used to calculate the habitual intake of dietary AGEs based on a FFQ. However, no detailed information about food preparation techniques, such as baking, grilling or frying, and also duration of this heating were described. Therefore, in ongoing studies we have implemented additional questions about food preparation and high AGE-content foods.

Overview of dicarbonyl compounds and AGEs in body fluids

With the developed techniques, described in this thesis, we were able to analyze dicarbonyl compounds, AGEs and related biomolecules in healthy and uremic patients, IBD patients and individuals with type 2 diabetes. An overview of the concentrations of these biomolecules in different disease states and body fluids, as presented in the different chapters of this thesis, is shown in table 10.1.

Tabel 10.1 Overview of dicarbonyl compounds and AGEs in body fluids.

Compound	Technique	Material	Subjects	Mean concentration
Pentosidine (protein-bound)	HPLC-fluorescence	Heparin plasma	Healthy volunteers: Uremic patients:	0.21 pmol/mg protein; median[10] 3.05 pmol/mg protein; median[10]
CML (protein-bound)	UPLC-MS/MS	EDTA plasma	Healthy volunteers: Uremic patients:	3129 nmol/L Chapter 2B 13832 nmol/L Chapter 2B
CML (free)	UPLC-MS/MS	EDTA plasma	Healthy volunteers: Uremic patients:	68.5 nmol/L Chapter 2B 1084 nmol/L Chapter 2B
CEL (protein-bound)	UPLC-MS/MS	EDTA plasma	Healthy volunteers: Uremic patients:	1902 nmol/L Chapter 2B 2762 nmol/L Chapter 2B
CEL (free)	UPLC-MS/MS	EDTA plasma	Healthy volunteers: Uremic patients:	104.5 nmol/L Chapter 2B 1345 nmol/L Chapter 2B
MG-H1 (protein-bound)	UPLC-MS/MS	EDTA plasma	Healthy volunteers: Uremic patients:	7800 nmo/L Chapter 2B 14105 nmol/L Chapter 2B
MG-H1 (free)	UPLC-MS/MS	EDTA plasma	Healthy volunteers: Uremic patients:	409.1 nmol/L Chapter 2B 4161 nmol/L Chapter 2B
L-Lactate	UPLC-MS/MS	Urine	Healthy volunteers: IBD* patients: T2DM** patients:	6.3 µmol/mmol creat[117] 11.8 µmol/mmol creat[117] 12.1 µmol/mmol creat[117]
D-Lactate	UPLC-MS/MS	Urine	Healthy volunteers: IBD* patients: T2DM** patients:	1.1 µmol/mmol creat[117] 3.1 µmol/mmol creat[117] 3.4 µmol/mmol creat[117]
L-Lactate	UPLC-MS/MS	EDTA plasma	Healthy volunteers: IBD* patients: T2DM** patients:	1044.8 µmol/L[117] 1172.4 µmol/L [117] 1534.7 µmol/L [117]
D-Lactate	UPLC-MS/MS	EDTA plasma	Healthy volunteers: IBD* patients: T2DM** patients:	8.0 µmol/L [117] 10.7 µmol/L [117] 12.3 µmol/L [117]
Methylglyoxal	UPLC-MS/MS	EDTA plasma	Healthy volunteers: T2DM** patients:	212 nmol/L[118] 277 nmol/L[118]
Glyoxal	UPLC-MS/MS	EDTA plasma	Healthy volunteers: T2DM** patients:	406 nmol/L[118] 514 nmol/L[118]
3-Deoxyglucosone	UPLC-MS/MS	EDTA plasma	Healthy volunteers: T2DM** patients:	1046 nmol/L[118] 2217 nmol/L[118]

*IBD: patients with inflammatory bowel disease
**T2DM: patients with type 2 diabetes mellitus

Quantitative research: a critical appraisal

In this thesis we have developed and used state-of-the-art techniques to quantify α-oxoaldehydes, AGEs and other biomolecules in the AGE-pathway, in a broad range of sample matrices, to investigate their role in relation to disease.

A large part of our medical and scientific knowledge is based on quantitative measurements. Quantitative data, of AGEs, are used in many studies to answer important research questions about disease and health. However, it is important that researchers, in this field, are aware of methodological limitations that could bias this data and influence the findings of their study. Quantitative data, of AGEs, may be biased due to a number of factors, including pre-analytical processes, sample preparation and matrix effects.

The first step in quantitative research is the collection of a given sample, such as blood, plasma or urine. This first step can be a major source of variability and artefacts due to the instability of the compounds of interest. Compounds can be subjected to oxidation, aggregation or degradation as a consequence of poorly defined pre-analytical procedures. Sample handling and storage (e.g. choice of anticoagulant and collection tubes, time of incubation before plasma separation from blood cells, storage conditions and freeze-thaw cycles) and their effect on the stability of the compounds of interest should be well investigated[119-121].

After collecting the sample it has to be prepared for analysis. Many sample preparation methods have been described, including various solvent precipitation techniques, ultra-filtration, liquid-liquid extraction and solid-phase extraction methods[25,36,122,123]. For the measurement of circulating molecules, such as free AGEs, D-lactate or α-oxoaldehydes in plasma, it is of utmost important to remove proteins by precipitation or ultra-filtration before analysis. The removal of these proteins prevents blockage of the LC-column and improves ion sufficiency during electrospray ionization detection[124]. On the contrary, protein-bound AGEs are determined after acid hydrolysis of the protein pellet and subsequently detected after derivatization. However, these sample preparation techniques can influence quantitative results by low metabolite coverage, low recovery and matrix effects[122].

In this thesis several analytical methods for the detection of AGEs, D-lactate and α-oxoaldehydes have been described. We have considered the methodological flaws, as described above. As described in previous chapters, stability, linearity, recovery and precision of the analytes were all well investigated and possible matrix effects were overcome by the use of stable-isotope internal standards.

Conclusions and future perspectives

Since the introduction of liquid chromatography (LC) by Martin and Synge[125] in 1941, many important improvements were done to make this technique the strong analytical tool as we know it today. Several manufacturers have improved hardware and software and pushed the applicability of LC to the next level. In this thesis the application and strength of high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) for the detection of dicarbonyls and AGEs were explored. With the developed techniques, we were able to further investigate the role of AGEs and related compounds in relation to disease. The main results of this thesis is the development of rapid, sensitive and selective LC and UPLC-tandem MS methods to analyze pentosidine, N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), L-lactate, D-lactate, Glyoxal (GO), Methylglyoxal (MGO) and 3-Deoxyglucosone (3-DG). With the developed techniques we found that:

- Plasma concentrations of protein-bound pentosidine, CML, CEL and MG-H1 and free CML, CEL and MG-H1 were significantly higher in uremic patients compared to healthy controls.

- Plasma D-lactate levels can be used as a reflection of MGO.

- Postprandial glucose excursions are related to an increase of α-oxoaldehyde levels in individuals with impaired glucose metabolism (IGM) and type 2 diabetes mellitus (T2DM).

- Higher intake of dietary protein-bound CML, CEL and MG-H1 is associated with higher levels of free plasma and urinary CML, CEL and MG-H1.

With the newly developed methods, as described in this thesis, we open the opportunity to investigate the glycation pathway in more detail. We already have shown that increased blood glucose levels, after postprandial glucose excursions or in patients with T2DM, plays an important role in the formation of α-oxoaldehydes. Consequently, these precursors can lead to an increased endogenous formation of AGEs and may explain the increased risk for micro- and macrovascular complications. Furthermore, exogenous sources of AGEs, such as dietary AGEs, can also contribute to the circulating AGEs. However, whether these dietary AGEs have important implications for those who ingest a diet high in AGEs is still under debate. To further investigate the consequences of these dietary AGEs, well designed intervention studies are needed.
In conclusion, the analytical methods and results as described in this thesis, are key, to obtain detailed information about the effects of dietary AGEs and endogenous AGEs for health and disease.

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SAMENVATTING

valorization

curriculum vitae

SCIENTIFIC OUTPUT

dankwoord

FOODS & POLAROID

Samenvatting

Samenvatting

Hart- en vaatziekten, obesitas en diabetes

Hart- en vaatziekten, obesitas en diabetes kunnen leiden tot gezondheids-problemen of een kortere levensverwachting. Wereldwijd hebben 1,9 miljard mensen overgewicht (hiervan hebben 600 miljoen mensen obesitas) en 422 miljoen mensen diabetes. In 2012 overleden 1,5 miljoen mensen aan de gevolgen van diabetes. In Nederland heeft ongeveer de helft van alle volwassenen overgewicht en leiden er 1 miljoen mensen aan diabetes. Dit aantal zal tot 2025 met 30% toenemen. Kortom, deze aantallen maken duidelijk dat wetenschappelijk onderzoek naar deze ziekten van groot belang is voor mens, maatschappij, gezondheid en economie.

Diabetes, ook wel suikerziekte, is een ziekte waarbij de suikerstofwisseling verstoord is. Via de voeding krijgt ons lichaam koolhydraten binnen in de vorm van suiker, zetmeel en vezels. Deze koolhydraten worden in ons lichaam omgezet naar glucose en worden in de weefsels (o.a. in de spieren) opgenomen en omgezet in energie. Het hormoon insuline, dat aangemaakt wordt in de alvleesklier, reguleert de opname van glucose in de weefsels. Dit mechanisme is verstoord bij mensen met diabetes. Er zijn twee soorten diabetes: type 1 en type 2. Bij type 1 diabetes (T1D) is er sprake van auto-immuundestructie van de β-cellen in de alvleesklier. Hierdoor is er een absoluut tekort aan insuline met als gevolg hoge bloedsuikerwaarden. Patiënten kunnen dan insuline spuiten om de bloedsuikerwaarden te normaliseren, bijvoorbeeld na een maaltijd. Ongeveer 10% van alle patiënten met diabetes heeft T1D. Bij type 2 diabetes (T2D) is er sprake van verminderde gevoeligheid voor insuline. Daarnaast wordt er ook minder insuline aangemaakt waardoor de bloedsuikerwaarde stijgt. Negentig procent van alle patiënten met T2D wordt veroorzaakt door leefstijlfactoren, zoals overgewicht en het gebrek aan fysieke activiteit. Mensen met diabetes hebben een 2 tot 4 keer hoger risico op het krijgen van hart- en vaatziekten ten opzichte van gezonde mensen. In het bijzonder, problemen aan de kleine bloedvaten (ogen, nieren en zenuwen) en aan de grote bloedvaten (hartaanval, beroerte en atherosclerose) zorgen voor dit toegenomen risico. Diabetes zorgt voor een permanente en significante vermindering van de kwaliteit van leven en voor een toename aan de kosten in de gezondheidszorg. Het is daarom van medisch en economisch belang dat diabetes en de daarmee gerelateerde hart- en vaatziekten voorkomen worden.

Diagnose & onderzoek

Vroege indicaties van een verhoogd risico voor hart- en vaatziekten bij patiënten met diabetes, en het toepassen van adequate interventies zal de negatieve gevolgen van deze ziekte sterk doen verlagen.
De enorme impact die hart- en vaatziekten met zich meebrengt dwingt ons tot het zoeken naar geschikte biomarkers, die voorspellend zijn voor hart- en vaatziekten in patiënten met diabetes. Echter, de traditionele cardiovasculaire risicofactoren zijn niet in staat het volledige mechanisme te verklaren. Een beter inzicht in de pathofysiologische routes en uitgebreid onderzoek naar hart- en vaatziekten bij mensen met (pre)diabetes is daarom noodzakelijk.

Advanced glycation endproducts (AGE's)

Versuikering van eiwitten, ook wel glycering genoemd, werd voor het eerst beschreven door de Franse arts en wetenschapper Louis Camille Maillard in 1912. Hij onderzocht de reactie tussen aminozuren en suiker en ontdekte dat deze reactie verantwoordelijk was voor de bruine kleur, specifieke smaak en geur bij de bereiding van voedsel. De naar hem vernoemde Maillard reactie is niet één simpele reactie tussen een aminozuur en suiker, maar een cascade van complexe reacties met uiteindelijk de vorming van advanced glycation endproducts (AGE's).
De vorming van deze AGE's vindt niet alleen plaats bij de bereiding van voedsel maar ook in het menselijk lichaam. Voornamelijk bij patiënten met diabetes, als gevolg van hoge bloedsuikerwaarden, maar ook als gevolg van een verminderde klaring, bij patiënten met chronische nierziekte. AGE's zijn in verband gebracht met diabetische complicaties en hart- en vaatziekten.

In het verleden werden immunochemische analyse technieken (met name enzyme-linked immunosorbent assays, ELISA's) gebruikt voor het kwantificeren van AGE's. Echter, de kwaliteit en betrouwbaarheid van deze methoden lieten te wensen over. Er moest dus gezocht worden naar gevoeligere en specifiekere technieken. Vloeistofchromatografie was een analysetechniek die al jaren succesvol werd toegepast voor het kwantificeren van biomoleculen. Deze techniek bleek ook uitermate geschikt te zijn voor de analyse van AGE's en andere aanverwante componenten. In dit proefschrift wordt een overzicht gegeven van de ontwikkeling en validatie van een aantal nieuwe chromatografische technieken alsook de toepassing van deze technieken in klinische studies voor de detectie van AGE's en zijn voorlopers.

Belangrijkste bevindingen uit dit proefschrift

In hoofdstuk 2A wordt een snelle en gevoelige vloeistofchromatografische methode beschreven voor het kwantificeren van pentosidine met fluorescentie als detectiemethode. De belangrijkste verbeteringen van deze nieuwe methode ten opzichte van de al beschreven methoden zijn: kortere runtijden, het gebruik van "reversed-phase'' chromatografie in plaats van ingewikkelde chromatografische systemen en een eenvoudige monstervoorbewerking. De beschreven methode werd succesvol toegepast voor het kwantificeren van eiwitgebonden pentosidine in plasma van gezonde vrijwilligers en patiënten met nierfalen. Er werd een significant hogere plasma pentosidine concentratie gevonden in de patiënten met nierfalen ten opzichte van gezonde vrijwilligers. Deze resultaten waren in overeenstemming met de literatuur.

Hoge druk vloeistofchromatografie gekoppeld aan massaspectrometrie is een analysetechniek die zijn waarde al in vele wetenschappelijke publicaties heeft bewezen. Voornamelijk de introductie en gebruik van zeer kleine partikels (<1.7 µm) bij het pakken van chromatografische kolommen heeft voor een enorme verbetering van de chromatografie gezorgd. De koppeling van deze scheidings-techniek (ultra performace liquid chromatography, UPLC) aan een zeer gevoelige en selectieve massaspectromter (tandem MS) resulteert in een van de beste analysetechnieken van dit moment.
In hoofdstuk 2B wordt deze techniek toegepast voor de analyse van drie eiwitgebonden en vrije AGE's in plasma, te weten: 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). Daarnaast was het met dit systeem ook mogelijk het aminozuur lysine te analyseren, dat gebruikt werd als een maat voor de hoeveelheid eiwit. Met deze techniek werd plasma onderzocht van gezonde vrijwilligers en patiënten met nierfalen. Zowel vrij- als eiwitgebonden plasma concentraties van CML, CEL en MG-H1 waren hoger in de patiënten met nierfalen ten opzichte van de gezonde vrijwilligers.

In hoofdstuk 3 werden de technieken, zoals beschreven in hoofdstuk 2A en 2B, gebruikt voor het onderzoeken van de samenhang tussen plasma AGE's en hart- en vaatziekten, in individuen met verschillende gradaties aan glucosetolerantie. Hiervoor werden eiwitgebonden plasma concentraties van CML, CEL en pentosidine gemeten, in twee Nederlandse cohort studies. Verassend genoeg werden er geen verbanden gevonden tussen de eiwitgebonden AGE's en hart- en vaatziekten. Daarnaast bleken eiwitgebonden plasma concentraties van CML, CEL en pentosidine, niet hoger te zijn in mensen met type 2 diabetes. Dit resultaat was in tegenstelling tot de eerder gepubliceerde data in mensen met type 1 diabetes.

Het derivatiseren van biomoleculen wordt vaak gebruikt om de polariteit te veranderen of het molecuul gevoeliger en toepasbaar te maken voor bepaalde detectietechnieken. In hoofdstuk 4 werd een chirale derivatisering, met diacetyl-L-wijsteenzuuranhydride, toegepast voor het scheiden van L- en D-lactaat met behulp van reversed-phase (RP)

chromatografie gekoppeld aan tandem MS. We waren voornamelijk geïnteresseerd in D-lactaat omdat dit het eindproduct is van het glyoxalase systeem, dat verantwoordelijk is voor de detoxificering van methylglyoxal. Met behulp van deze methode werden concentraties van L- en D-lactaat gekwantificeerd in plasma en urine van gezonde vrijwilligers, patiënten met inflammatoire darmziekten en patiënten met T2D. Plasma en urine L- en D-lactaat concentraties waren significant hoger in patiënten met T2D. In patiënten met inflammatoire darmziekten werd alleen een significant hogere concentratie aan urine L-lactaat gevonden ten opzichte van gezonde vrijwilligers. Deze resultaten bevestigen dat D-lactaat een reflectie is van de detoxificatie van methylglyoxaal door het glyoxalase systeem en niet van de D-lactaat productie in de darmen.

In hoofdstuk 5 werd de samenhang van plasma D-lactaat concentraties met insuline resistentie onderzocht. Hiervoor werd cross-sectioneel onderzoek gedaan in het CODAM cohort (cohort study on diabetes and atherosclerosis Maastricht). Er werd een positieve associatie gevonden tussen plasma D-lactaat en insuline resistentie, onafhankelijk van potentiële confounders. Deze resultaten suggereren dat methylglyoxal een rol speelt in insuline resistentie. Echter, om dit te bevestigen zal verder onderzoek noodzakelijk zijn met specifieke interventies.

In hoofdstuk 4 en 5 werd reeds gespeculeerd over de mogelijke rol van methylglyoxaal bij het ontwikkelen van insuline resistentie. Om deze resultaten te bevestigen en verder te onderzoeken werd in hoofdstuk 6 een nieuwe en verbeterde methode opgezet voor het analyseren van drie α-oxoaldehydes, te weten: glyoxaal, methylglyoxaal en 3-deoxyglucosone. Daarnaast werd een uitgebreide validatie van de methode en monstervoorbewerking uitgevoerd. Om stabiele en betrouwbare concentraties van deze α-oxoaldehydes te verkrijgen vonden we dat het bloed afgenomen dient te worden in EDTA buizen en een directe onteiwiting met perchloorzuur noodzakelijk is om de α-oxoaldehyde concentraties te stabiliseren. Met de beschreven methode werden verhoogde plasma α-oxoaldehyde concentraties gevonden in patiënten met T2D ten opzichte van gezonde controles.

In hoofdstuk 7 werd de invloed van een orale glucose belasting op plasma α-oxoaldehyde concentraties onderzocht. Hiervoor werd cross-sectioneel onderzoek gedaan in het CODAM cohort. Plasma concentraties van glyoxaal, methylglyoxaal en 3-deoxyglucosone werden gemeten in de nuchtere toestand en na een orale glucose tolerantie test (OGTT). Het hebben van prediabetes en type 2 diabetes was geassocieerd met hogere plasma α-oxoaldehyde concentraties gedurende de OGTT. Deze resultaten tonen aan dat er een stijging is van dicarbonyl stress in voornamelijk patiënten met (pre)diabetes na het nuttigen van glusose-houdende dranken.
Deze verhoogde productie heeft mogelijk ook consequenties voor de vorming van AGE's. Echter, de endogene vorming van AGE's is veel minder snel dan de vorming van α-oxoaldehydes en zal daardoor waarschijnlijk geen acuut effect laten zien.

In de inleiding werd beschreven dat AGE's aanwezig zijn in voedsel, voornamelijk bij de bereiding (bakken, grillen en frituren) van voedsel worden deze AGE's gevormd. Mogelijk leveren deze in voeding aanwezige AGE's, wel een directe bijdrage op de concentratie van circulerende AGE's in plasma. Om dit te onderzoeken dient men inzicht te krijgen in de hoeveelheid AGE's per voedingsproduct. In hoofdstuk 8 werd daarom een UPLC-tandem MS methode ontwikkeld voor het analyseren van AGE's in voedingsproducten geconsumeerd in een westers dieet. Eiwitgebonden CML, CEL en MG-H1 concentraties werden in 190 verschillende voedingsproducten gekwantificeerd. Fruit, groente, boter en koffie waren producten met een lage of verwaarloosbare AGE concentratie. Hoge AGE concentraties werden gevonden in gebakken, gefrituurde en gegrilde producten, zoals vlees, pindakaas, frikadel en noten.

In hoofdstuk 9 werd de voedings-AGE-database, zoals beschreven in hoofdstuk 8, gebruikt om de associatie tussen ingenomen AGE's (via de voeding) en circulerende AGE's te onderzoeken. Plasma en urine concentraties van CML, CEL en MG-H1 werden geanalyseerd in 465 individuen van het CODAM cohort. Daarnaast werd de AGE-inname geschat aan de hand van een voedingsvragenlijst en de AGE-database. De conclusie van dit onderzoek was, dat het eten van voedsel rijk aan AGE's geassocieerd is met de concentraties van vrije AGE's, zoals gemeten in plasma en urine. Deze resultaten duiden op de mogelijke consequenties van het eten van een dieet rijk aan AGE's.

Conclusie

Dit proefschrift beschrijft de toepassing van nieuwe analysetechnieken voor het onderzoeken van AGE's en zijn voorlopers in diverse matrices. De belangrijkste conclusie van dit proefschrift is dat vloeistofchromatografie gekoppeld aan massaspectrometrie een waardevolle en zeer betrouwbare methode is voor het analyseren van biomoleculen in complexe matrices. Met deze techniek werd aangetoond dat AGE's en zijn voorlopers nauw verband houden met verschillende ziekten, zoals diabetes, nierziekte, hart- en vaatziekten en inflammatoire darmziekte. Echter, het causale verband tussen AGE's en deze ziekten zal nog verder onderzocht moeten worden. Hiervoor zullen specifieke interventiestudies noodzakelijk zijn, waarbij gekeken wordt naar het remmen van AGE's of verlaging van AGE's vanuit de voeding en de invloed hiervan op bijvoorbeeld insuline resistentie of het ontwikkelen van hart- en vaatziekten. De toepassing van de technieken, beschreven in dit proefschrift, zullen een noodzakelijke rol spelen in dit vervolgonderzoek. Daarnaast staan de ontwikkeling van nieuwe technieken voor het analyseren van andere AGE's en biomoleculen niet stil. De zoektocht naar geschikte biomarkers, voor het voorspellen van ziekten en complicaties en daarmee de behandelingsstrategie te verbeteren, zal de inzet zijn van toekomstig onderzoek.

Valorization

Valorization

More than 2000 years ago, Hippocrates, a well-known Greek physician, was one of the founding fathers of the modern pathology. He developed new methods to examine the human body and was one of the first who used body fluids (blood, phlegm, yellow bile and black bile) in order to understand the etiology and pathogenesis of a number of diseases. Today, researchers are still using body fluids (such as blood, plasma, serum, feces, saliva and urine) to investigate and understand diseases. One way of doing these investigations, is using measureable indicators to examine the physiological state of an individual. These indicators, also known as (disease-related) biomarkers, are a valuable tool to understand the mechanisms, and even more important, treat and prevent many diseases. Sensitive, specific and reliable state-of-the-art techniques are necessary to determine these biomarkers in many different matrices. In the present thesis we validated and developed new analytical methods to quantify specific biomarkers in order to investigate diabetes and cardiovascular disease (CVD).

Relevance of this thesis

In 2012, an estimated 1.5 million deaths were directly caused by diabetes[1] and even 17.5 million people died from CVD[2]. Individuals with diabetes are at an increased risk of cardiovascular events[3,4] and its associated complications leads to a permanent and significant loss of quality of life. Therefore, prevention of diabetes and in case of diabetes, prevention of cardiovascular disease, is of highest importance and clearly represents a medical and economical need[5]. Future strategies should therefore focus primarily on the prevention of diabetes. Given the enormous impact of cardiovascular disease, it is of utmost importance to find biomarkers for the identification of patients with diabetes which are at high risk of developing vascular complications and morbidity; i.e. to improve risk prediction[6]. In this thesis we focused on the analysis of advanced glycation endproducts (AGEs) and related biomarkers in relation to diabetes and CVD.

Advanced glycation endproducts and disease

AGEs are formed by the reaction of proteins with reactive metabolic intermediates derived from glucose and from lipid oxidation with the involvement of oxidative stress[7-9]. Increased endogenous formation and accumulation of AGEs is common in patients with diabetes, and, because of impaired clearance, in those with chronic kidney disease (CKD)[10] or end stage renal disease (ESRD)[11]. AGEs are implicated to play a role in the development of vascular disease[12-17], age-related disease[13,18,19] and diabetes[20]. Most recent, it has become apparent that dietary AGEs represent a significant source of circulating and tissue AGEs[21-25].

However, many of these AGE-related researches were based on techniques, such as enzyme-linked immunosorbent assay (ELISA). However, new and improved analytical techniques are necessary in order to investigate the role of these AGEs in disease.

Applications of biomarker analysis

In this thesis we have used ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) to determine AGEs and other related biomarkers, such as methylglyoxal, in healthy individuals, patients with type 2 diabetes mellitus (T2DM), patients with impaired glucose metabolism (IGM), patients with inflammatory bowel disease (IBD) and patients with end stage renal disease (ESRD). Moreover, this technique was used to determine the AGE content in foods. AGEs have traditionally been detected by ELISA[26-32]. For several reasons the use of antisera for quantitative immunoassays of protein-bound AGEs is questionable. Reproducibility and sensitivity of such an assay are not optimal, because the specificity of the antibodies is often difficult to define and, because of steric constraints, not all AGE epitopes on the protein may be available for interaction with the antibody [28,30]. Thus, AGE measurements with immunoassays should be interpreted with care. A better approach for the quantitative determination of specific AGEs is the use of a specific analytical technique. For the determination of AGE levels in both tissue and blood samples, high performance liquid chromatography (HPLC) measurements[33-36] and several mass spectrometry methods have been developed including gas chromatography mass spectrometry (GC–MS)[37,38], and liquid chromatography tandem mass spectrometry (LC–MS/MS)[32,36,39,40]. However, the simultaneous quantification of different AGEs in plasma or tissue, using a highly sensitive, selective and rapid analytical method, has not been reported yet.

In chapter 2, 4, 6 and 8 state-of-the-art techniques were described to analyze pentosidine, N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), 5-hydro-5-methylimidazolone (MG-H1), L-lactate, D-lactate, methylglyoxal, glyoxal and 3-deoxyglucosone. These techniques were successfully applied to investigate disease mechanisms.

In chapter 5 and 7 we describe the potential role of the biomarkers D-lactate and methylglyoxal in insulin resistance and the increased risk of diabetic complications. We found increased plasma concentrations of α-dicarbonyls and D-lactate in patients with T2DM as compared to non-diabetic controls. Moreover, an oral glucose tolerance test (OGTT) was performed in individuals with normal glucose metabolism (NGM), IGM and T2DM. The area under the curve (AUC) for the OGTT levels of the α-dicarbonyls and glucose were higher in individuals with IGM and T2DM as compared with NGM.

These findings underline the potential importance of α-dicarbonyl stress as a candidate to explain the increased risk of diabetic complications in individuals with postprandial hyperglycemia.

In chapter 8 we validated a new method to determine dietary AGEs (CML, CEL and MG-H1) in 190 food products as consumed in a Western diet. A dietary AGE database was presented and applied to calculate the dietary AGE intake of a Dutch cohort. In chapter 9 we used these data and demonstrated that higher intake of dietary AGEs is associated with higher levels of plasma and urinary AGEs. These findings are an important step in understanding the metabolic transit of these dietary AGEs. Several studies indicate that dietary AGEs are associated with poor health[13-15,23,29,42]. Future prospective studies should address whether dietary AGEs are associated with adverse outcomes such as accelerated development of cardiovascular disease, diabetes and other age-related diseases.

Future perspective

In the following years the UPLC-MS/MS applications, described in this thesis, will be used in many different studies. They create opportunities to investigate not only the glycation pathway but also other disease mechanisms in more detail. However, as new research questions arise, analytical methods will need to evolve as well. In this thesis we have made the first step in investigating the complex biochemical pathway of glycation and related mechanisms. We have shown that glucose is the key molecule which is responsible for initiating glycation and formation of reactive α-dicarbonyls. We have also shown that dietary AGEs are influencing circulating AGEs. However, several questions need to be answered: 1. What is the effect of AGEs for human health? and 2. Do dietary AGEs play a significant role in developing diseases, such as diabetes and CVD? To answer these questions, the effect of AGE inhibitors and AGE-diet interventions need to be investigated. These studies are ongoing. Biomolecule analysis with state-of-the-art UPLC-MS/MS techniques, such as the one described int this thesis, will be a fundamental part in these studies.

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Curriculum vitae

Curriculum vitae

Jean Scheijen was born on August 29th, 1971 in Heerlen and raised in Schin op Geul (the Netherlands). He graduated from secondary school (Ravensberg College, Valkenburg aan de Geul) in 1987 and started in that same year his study Analytical Chemistry at the Walram College in Sittard. During this period he did an internship at the Laboratory of Rolith Chemicals (Roermond) where he was responsible for the quality control of the production line.

After graduation in 1992, he started studying Environmental Chemistry at the *Hogeschool Limburg* in Sittard and graduated in 1997. During this period he did an internship and master project at the Laboratory of Clinical Pharmacology and Toxicology (Maastricht University Medical Centre, *MUMC+*).

Hereafter, he started working at the Laboratory of Inborn Errors of Metabolism (1998, Clinical Genetics, *MUMC+*). After one year he was offered a job at the Department of General Surgery (Maastricht University) and obtained here his first experience with Liquid Chromatography Mass Spectrometry (LC-MS). For five years he worked under the supervision of Mick Deutz and Hans van Eijk at this department.

In 2003, the department of Clinical Genetics wanted to implement new analytical techniques to investigate inborn errors. Together with Huub Waterval, Jean Scheijen implemented two LC-MS systems at the Laboratory of Inborn Errors and succeeded in developing new methods to analyze several biomolecules to investigate metabolic disorders. These methods are used until this day.

After five years, he was asked to set up an analytical unit at the Laboratory of Metabolism and Vascular Medicine (Internal Medicine, Maastricht University, *MUMC+*). Under the supervision of Casper Schalkwijk and Coen Stehouwer he implemented several LC and LC-MS methods to investigate diabetes and cardiovascular disease. In 2013, he started his PhD at that same department and continued working there as a Senior Research Technician.

Scientific output

Scientific output

List of publications

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Bloed, zweet en tranen

Bloed, zweet en tranen

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2. Prof. dr. C.D.A. Stehouwer, Internist (Maastricht Universitair Medisch Centrum plus), hoofd Interne Geneeskunde, bijzonder hoogleraar Interne Geneeskunde

Foods & Polaroid

Chapter title pages
Original Polaroid & Impossible Project film

BANANA

Film: Original Polaroid 600 with ND filter
Camera: Polaroid SX-70 Sonar AF

CAPPUCCINO

Film: Original Polaroid 600 with ND filter
Camera: Polaroid SX-70 Original

COLA

Film: Original Polaroid 600 with ND filter
Camera: Polaroid SX-70 Original

HEINZ TOMATO KETCHUP

Film: PX-70 Color Shade (Impossible Project)
Camera: Polaroid SX-70 Alpha 1 model 2

PEPPER

Film: PX-70 Color Shade (Impossible Project)
Camera: Polaroid SX-70 Original

WINE

Film: PX-70 Color Shade (Impossible Project)
Camera: Polaroid SX-70 Original

CORONITA & OLIVE OIL

Film: Original Polaroid 600 with ND filter
Camera: Polaroid SX-70 Alpha 1 model 2

FASTFOOD

Film: Original Polaroid 600 with ND filter
Camera: Polaroid SX-70 Original

ICE CREAM (Giel & Pie)
Film: Polaroid Image Softtone
Camera: Polaroid Image System

VEGETABLES
Film: Polaroid Image Softtone
Camera: Polaroid Image System

MERLOT
Film: PX-70 Color Shade (Impossible Project)
Camera: Polaroid SX-70 Original

MARTINI & GIN
Film: PX-70 Color Shade (Impossible Project)
Camera: Polaroid SX-70 Alpha 1 model 2