Methylglyoxal, the glyoxalase pathway and advanced glycation endproducts in type 2 diabetes and cardiovascular disease

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**Methylglyoxal, the glyoxalase pathway and
advanced glycation endproducts in type 2
diabetes and cardiovascular disease**

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Methylglyoxal, the glyoxalase pathway and advanced glycation endproducts in type 2 diabetes and cardiovascular disease

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Contents

Chapter 1	General introduction	7
Chapter 2	Associations between the ankle-brachial index and cardiovascular and all-cause mortality are similar in individuals without and with type 2 diabetes - 19-year follow-up of a population-based cohort study	27
Chapter 3	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	39
Chapter 4	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	55
Chapter 5	Circulating GLO1 mRNA and plasma D-lactate levels are higher in type 2 diabetes, but are not associated with markers of atherosclerosis and presence of cardiovascular disease: the CODAM study	75
Chapter 6	Plasma advanced glycation endproducts are associated with incident cardiovascular disease in individuals with type 2 diabetes: a case-cohort study with a median follow-up of 10 years (EPIC-NL)	93
Chapter 7	Higher levels of advanced glycation endproducts in human carotid atherosclerotic plaques are associated with a rupture-prone phenotype	113
Chapter 8	Glyoxalase 1 overexpression does not affect atherosclerotic lesion size and severity in ApoE ^{-/-} mice with or without diabetes	149
Chapter 9	Summary and main findings, discussion,	175
	Samenvatting	192
	Dankwoord	201
	Curriculum Vitae	209
	Abbreviations	215

Chapter 1

General introduction

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Diabetes and cardiovascular disease

The ever rising prevalence of diabetes represents one of the major challenges in modern health care. A staggering 382 million people are now afflicted by this disease worldwide, and its prevalence is expected to reach 600 million in 2035.

Diabetes is characterised by increased plasma glucose levels, and is the result of defective insulin secretion and/or -signalling. Most people suffer from type 2 diabetes mellitus (T2DM), which usually affects older and/or obese individuals, while approximately 10% of individuals with diabetes have type 1 diabetes, which is an auto-immune disease of the insulin-producing cells in the pancreas.¹ Although especially people with T2DM may have few symptoms initially, diabetes is by no means a harmless disease. Having diabetes may lead to several severe complications, such as diabetic nephropathy, a leading cause of needing dialysis; diabetic retinopathy, a leading cause of blindness; and to diabetic neuropathy and diabetic ulcers of the feet. Unfortunately, diabetes also predisposes people to many other chronic diseases, such as depression, dementia, and in particular cardiovascular disease (CVD), of which heart attack and stroke are common examples. In fact, CVD is the major cause of mortality in people with diabetes.² For example, individuals with T2DM have an equally high risk of cardiovascular death as individuals who had a heart attack.³ Furthermore, although the major hallmark of diabetes is an increase in glucose levels, glucose-lowering treatment does not lower risk of CVD quickly.⁴ This phenomenon is often called the “glucose paradox”.⁵ Therefore, the increased development of CVD by diabetes is still not completely understood.

The process underlying the vast majority of CVD is atherosclerosis, which is hallmarked by build-up of atherosclerotic plaques in the arterial vessel wall. An atherosclerotic plaque is formed by accumulation of lipids (particularly low-density cholesterol (LDL) particles) and inflammatory cells between the medial and intimal layers of arteries (Figure 1.1A). Advanced atherosclerotic plaques consist of a necrotic core containing cholesterol and dead macrophages, flanked by macrophage-rich shoulder regions, and are covered by a fibrous cap containing smooth muscle cells and collagen. Stable plaques are characterized by more collagen deposition, smooth muscle cells, fewer macrophages and a small necrotic core.⁶ Plaques at highest risk of rupture are rich in macrophages and have a large necrotic core (Figure 1.1B). In addition, infiltration of leaky intra-plaque vessels contributes to intra-plaque hemorrhages, increasing the risk of plaque rupture.⁷ Ruptured plaques are defined by a disruption of the fibrous cap and a thrombus that is continuous with the necrotic core, which may occlude an artery.⁷ The risk of plaque rupture is thought to be a balance between inflammatory activity and growth of the necrotic core versus thickness of the fibrous cap.^{8,9} It is unknown why in some plaques the fibrotic processes dominate, leading to stable plaques, while in other plaques inflammation and necrosis take over, leading to thinning of the fibrotic cap. Insight into this process

could yield new treatments preventing plaque rupture, or could lead to new methods to identify people at increased risk of CVD, people with diabetes for example.

Figure 1.1 Panel A: Overview of the layers forming a healthy artery. Panel B: Accumulation of AGEs in atherosclerotic plaques may contribute to progression from stable to ruptured plaques. Figure adapted from Watkins et al. (Nature genetics, 2006).

Previous studies have proposed high metabolic activity in atherosclerotic plaques may contribute to the development of plaque rupture.¹⁰ One feature of increased metabolic activity is formation of advanced glycation endproducts (AGEs).¹¹ Indeed, several studies have shown that AGEs accumulate in human atherosclerotic plaques.¹²⁻¹⁵ AGEs are a large family of extensively and irreversibly sugar-modified proteins, but rather than glucose itself, the dicarbonyl methylglyoxal (MGO) has been identified as the most reactive AGE precursor¹⁶ (Figure 1.2). MGO has attracted a lot of attention as a key player in vascular dysfunction, particularly due to its capacity to induce oxidative stress, cell death and endothelial dysfunction.^{16,17} Therefore, accumulation of MGO and MGO-derived AGEs may be a major factor contributing to plaque rupture. Since MGO is detoxified by the glyoxalase system, this may be a major protective factor against plaque rupture. These hypotheses are further explored in this thesis.

Advanced glycation endproducts (AGEs)

The formation of AGEs is complex, may arise from many distinct metabolic pathways, and many AGEs presumably have not even been discovered yet. Figure 1.2 shows an overview of the major pathways through which AGEs may be formed, and these will now be explained in further detail.

Figure 1.2 Simplified overview of the routes of formation for markers of the major known glycation pathways. Please note that this figure only includes AGEs, AGE precursors, and markers of AGE detoxification measured in the current thesis. Many more AGEs exist. Presumably, most of them have not even been discovered yet.

The Maillard reaction

Formation of AGEs was first described by Louis Camille Maillard in 1912.¹⁸ The Maillard reaction is initiated by the non-enzymatic reaction of reducing sugars with protein residues, resulting first in the formation of a Schiff base and subsequently, by structural rearrangements, in Amadori products. Only a small proportion of Amadori products are further, and irreversibly, modified to form AGEs. In the body, this entire reaction-chain occurs slowly over a period of months. Therefore, AGEs derived from the Maillard reaction are mostly formed on long-lived proteins, such as on collagen.¹⁹ A well-known AGE derived from this reaction is pentosidin. It is thought that the formation of AGEs is to a certain extent an inevitable consequence of (human)

biology, associated with aging.¹⁹ However, increased metabolic activity, such as in diabetes, is thought to accelerate AGE formation.²⁰

AGE formation via methylglyoxal and the glyoxalase system

It is now well established that intermediates of glycolysis are much more potent glycating agents than glucose itself,²¹ suggesting that *in vivo* formation of AGEs intracellularly is more abundant than AGE formation via the slow Maillard reaction on connective tissues. During glycolysis, highly reactive intermediates are formed, including the dicarbonyl compound MGO.¹⁶ MGO reacts primarily with arginine residues to form the AGEs 5-hydro-5-methylimidazolone (MG-H1), argpyrimidine and tetrahydroimidazolone (THP) as well as with lysine residues, to form N^ε-(carboxyethyl)lysine (CEL).

AGE formation via lipid peroxidation

In addition to formation of AGEs from glucose and glycolysis-derived precursors, AGEs can also be derived from lipid-peroxidation, of which the AGE N^ε-(carboxymethyl)lysine (CML) is a well-characterised example.²² These modifications are also often referred to as Advanced Lipid peroxidation Endproducts (ALEs). The mechanism of formation is similar to the MGO derived AGEs, where the oxidation of lipids leads to accumulation of highly reactive intermediates, which when they react with amino acids form AGEs/ALEs. The AGE precursor glyoxal (GO) is a well-known precursor for AGE/ALE formation.²³ Interestingly, the LDL particle is especially sensitive to modification through lipid peroxidation.²³

Endogenous protection from AGEs

MGO can be detoxified by glyoxalase 1 (GLO1) and GLO2 and reduced glutathione into D-lactate,²⁴ thereby preventing accumulation of MGO and MGO-derived AGEs (Figure 1.2). Overexpression of GLO1, the rate-limiting enzyme of the glyoxalase system, has been shown to reduce accumulation of MGO and AGEs in diabetes,²⁰ and protects against vascular dysfunction,²⁵ retinopathy²⁶ and nephropathy²⁵ in diabetic rats. MGO and AGE formation has mainly been studied in the context of hyperglycaemia, although inflammation and hypoxia may also be major determinants of MGO formation as glycolysis is increased under these conditions as well.

AGEs in atherosclerosis

Several studies have described AGE accumulation in human atherosclerotic plaques of the aorta,¹² coronary artery,¹³ carotid artery¹⁴ and femoral artery,¹⁵ using antibodies against glycated proteins¹²⁻¹⁴ or the well characterized AGE CML.^{15,27,28} Therefore,

accumulation of AGEs in atherosclerotic lesions seems irrespective of the site of plaque development. The specific MGO-derived AGE THP²⁹ has also been detected in atherosclerotic plaques. Furthermore, in line with their high metabolic activity, macrophages are the predominant cells in which the major AGEs CML and MG-H1 accumulate in plaques, particularly surrounding the necrotic core (see Figure 1.3A,B). Additionally, staining of these AGEs is also found in plaque vessels (Figure 1.3C,D).

Figure 1.3 Immunohistochemical staining of advanced carotid atherosclerotic lesions, showing staining of CML (A) and MG-H1(B) in the cytoplasm of macrophages (A,B indicated by black arrows) surrounding the necrotic core (indicated by black line). In addition, CML (C) and MG-H1 (D) accumulate in plaque vessels (indicated by white arrows). Magnification is 200x.

Factors contributing to AGE accumulation in atherosclerosis

Several metabolic factors such as inflammation, hypoxia and oxidative stress may promote an increased accumulation of AGEs in atherosclerotic plaques, while defensive mechanisms against glycation may be decreased.

AGE formation via glycolysis

In human atherosclerotic plaques, ¹⁸Fluor-deoxyglucose uptake experiments have shown that the macrophage is the primary cell in which a high degree of glycolysis takes place.¹⁰ It has been demonstrated in cultured human macrophages that inflammation is a stronger trigger for glucose uptake than hyperglycaemia.³⁰ The uptake of glucose under these circumstances may contribute to increased formation of MGO. In endothelium, hyperglycaemia leads to rapid accumulation of AGE precursors, including MGO.²¹ Furthermore, accumulation of AGEs in endothelial cells has been linked to atherosclerotic lesion initiation in diabetes.³¹

Lipid-derived AGE accumulation

Since macrophages take up large quantities of oxidized LDL in atherosclerotic lesions, AGEs derived from lipid peroxidation in macrophages may also be of great physiological importance in atherosclerosis. In addition, it has been demonstrated that LDL itself is a target for MGO. Modification of LDL by MGO decreases its particle size,³² increasing its atherogenicity, and reduces its affinity for the LDL-receptor,³³ thereby decreasing its clearance from the circulation and promoting trapping of LDL in the vessel wall.

Role of Glyoxalase 1 in preventing atherosclerosis is unclear

GLO1 is abundantly present in the cytosol of virtually all cells in the plaque (Figure 1.4). However, previous experimental studies have shown decreased GLO1 activity under conditions of metabolic stress, such as hyperglycaemia³⁴ and ischemia.³⁵ Given the high metabolic activity in atherosclerotic plaques, downregulation of GLO1 may lead to increased glycation in the plaque. Previous work has showed that the promoter of GLO1 harbours a NF- κ B responsive element,³⁶ indicating a link between inflammation and GLO1 expression. In addition, the GLO1 protein is susceptible to post-translational modifications, in particular phosphorylation in response to tumour necrosis factor, a key mediator of inflammation.³⁷ Moreover, posttranslational modification of GLO1 by oxidised glutathione and nitrosylation strongly inhibits GLO1 activity.^{38,39} The presence of such modifications on GLO1 in the plaque remains unclear. GLO1 is absent in the necrotic core of atherosclerotic plaques (Figure 1.4). Therefore, it is not conceivable that GLO1 offers full protection against extracellular AGE formation in advanced atherosclerotic plaques, such as AGE formation from lipid oxidation of LDL particles.

GLO1, 40X

Figure 1.4 Immunohistochemical staining of an advanced carotid atherosclerotic lesion, showing abundant staining of GLO1 in the cytoplasm of cells throughout the plaque, except for the necrotic core (indicated by black line). Magnification is 40x.

Potential mechanisms of how MGO and AGEs contribute to plaque rupture

Two major mechanisms by which AGEs damage tissues and contribute to the initiation, progression, and rupture of plaques can be proposed: firstly, intracellular glycation of proteins, leading to impaired cell function, and secondly, binding of AGEs to cellular receptors and subsequent modulation of inflammatory gene expression.

Cytotoxicity of MGO

Accumulation of MGO in macrophages may contribute to growth of the necrotic core of rupture-prone plaques, by induction of apoptosis and/or necrosis. *In -vitro*, MGO induces apoptosis of macrophages in an oxidative stress-dependent mechanism.⁴⁰ Accumulation of MGO and AGEs in plaque vessels may contribute to their dysfunction and leakage, further exacerbating their risk of intra-plaque hemorrhage and subsequent plaque rupture. Accordingly, MGO impairs proteasome function in endothelial cells,⁴¹ and indeed induces apoptosis.⁴²

Furthermore, it has been demonstrated that intracellular accumulation of MGO in endothelial cells causes dysfunction as indicated by expression of adhesion molecules

such as VCAM-1 expression, which can be prevented by GLO1 overexpression.⁴³ Therefore, AGE accumulation in endothelial cells may contribute to atherosclerotic plaque formation by attracting more monocytes to the plaque. Interestingly, El-Osta et al. demonstrated elevated MCP-1 expression and concomitant adverse epigenetic changes in endothelial cells of even normoglycaemic GLO1 knockdown mice,⁴⁴ suggesting that even transient exposure to MGO leads to a prolonged risk of atherosclerosis.

Inflammation through interaction with the receptor for AGEs

The receptor for AGEs (RAGE) links the accumulation of AGEs with inflammatory pathways associated with atherosclerosis. Activation of RAGE leads to activation of NF- κ B⁴⁵ and downstream inflammatory signaling. Furthermore, genetic deletion of RAGE greatly reduces atherosclerosis in ApoE^{-/-} mice.⁴⁶ In addition, administration of recombinant soluble RAGE reduced atherosclerosis in streptozotocin (STZ) injected diabetic ApoE^{-/-} mice. Moreover, in human plaques, RAGE co-localizes with MMP-9, a major collagen digesting enzyme, and RAGE signaling is implicated in weakening of the fibrous cap of the plaque.⁴⁷ CML is present in high amount in atherosclerosis and is considered an important ligand for RAGE.⁴⁸ However, many inflammatory cytokines, such as S100b and high motility group box 1⁴⁹ are ligands for RAGE, and which of these is the major ligand for RAGE in the plaque is not clear. Interestingly, an interplay between RAGE and the glyoxalase system has been described, as GLO1 overexpression prevents RAGE upregulation.⁵⁰

Measurement of cardiovascular disease and atherosclerosis in large cohort studies

Adequate measurement of CVD is very important in studying associations between plasma AGEs and CVD in large cohort studies, a major aim of this thesis. CVD can be assessed in several ways. By far the easiest approach is simply asking study participants if they have a history of CVD and/or verifying this against hospital records of the study participant. Although a history of CVD is indeed a robust predictor of developing a new CVD event, after a person developed CVD he/she usually receives treatment, which may mask associations between the biomarker of interest with prior CVD. This is especially problematic if the event occurred a long time ago. In addition, it is only possible to investigate non-fatal CVD through this approach.

Incident CVD (recording new events) is the gold standard for assessing associations with CVD. Data on incident CVD are obtained by recording when people suffered new CVD events through systematic enquiry of the hospital records of the study participant at a time point *after* he/she is included in the study. Likewise, occurrence

of fatal CVD can be recorded by systematic enquiry of the mortality register of the municipality where the study takes place. This approach is less susceptible to the limitations relevant for prior CVD. Unfortunately, there are some other limitations to recording incident CVD. Firstly, this approach requires follow-up on study participants for a very long time and is therefore costly and time-consuming. Secondly, since CVD events are luckily relatively rare at a younger age, quite large numbers of study participants need to be included to record a sufficient amount of cases. Furthermore, incident CVD tends to exclude people with asymptomatic CVD (or people that did not seek treatment).

Alternatively, it can be attempted to not measure CVD events directly, but perform measurements that reflect the development of either the underlying atherosclerosis and/or the processes that lead up to the CVD event. A well-known marker of atherosclerosis is for example carotid intima media thickness (cIMT).⁵¹ This is an ultrasound-based technique that measures thickness of the intima and media of the carotid artery as a presumed marker for atherosclerotic lesion size. Another well-known marker of widespread atherosclerosis is the ankle-brachial index (ABI). The ABI is a relatively simple method to assess systemic atherosclerosis, and is obtained by measuring blood pressure in both the ankles and arms, and dividing the ankle blood pressures by the highest arm blood pressure. The lowest value of either leg is used. Since the presence of atherosclerotic plaques lowers blood pressure in the ankle by obstructing bloodflow, a lower ABI value is assumed to be associated with a higher atherosclerotic burden, and an ABI value below 0.9 is generally considered abnormal.⁵²

Major advantages of markers like cIMT or ABI are that they are less susceptible to failure of recalling or recording CVD events, and often require less study participants because they are measured on a continuous scale. An additional advantage is that they allow the study of asymptomatic CVD. A major limitation is that these markers may not only reflect the actual disease the investigator is interested in, but may also include other pathological processes, and therefore the validity of these markers must be questioned carefully. This is particularly the case for the ABI, as it became apparent that not only a low (≤ 0.9), but also a high value (≥ 1.4) of the ankle-brachial index is associated with a higher risk of CVD.⁵² It is thought that the high ABI values are explained by calcification of arteries,⁵³ a condition that occurs more frequently in diabetes. If atherosclerosis and arterial calcification coincide, this may lead to a falsely normal ABI. Therefore, the ABI may be less reliable in diabetes, as both atherosclerosis and arterial calcification occur more frequently in diabetes. Because the ABI was measured in both individuals with- and without diabetes in some of the studies included in this thesis, this issue is addressed in this thesis (**Chapter 2**).

Reliable measurements of AGEs

Observational studies into the specific role of AGEs in diabetes and CVD have long been hampered by absence of reliable measurement tools to quantify AGEs. As AGE formation is a very heterogeneous process, previous ELISA-based techniques, using antibodies against glycated proteins have been proven not to be very specific, and even ELISAs based on antibodies against specific AGEs such as CML yield only semi-quantitative results. In addition, ELISA cannot distinguish between protein-bound and free fractions of circulating AGE modifications. In fact, AGE-precursors, which are not proteins, cannot even be detected with ELISA. In this thesis, several AGEs and AGE precursors were measured with tandem-mass ultra performance liquid chromatography (UPLC-MS/MS), and the AGE pentosidine, which has fluorescent properties, was measured with high performance liquid chromatography (HPLC).

Figure 1.5 Panel A shows the ultra-performance liquid chromatographer we have used in our studies for the detection of D-lactate, dicarbonyls and most AGEs. Panel B shows an example of a chromatogram, in this case of 3-deoxyglucosone, glyoxal and methylglyoxal standard (BA), pooled EDTA plasma (BB) and the internal standards for these components (BC). Please note that methylglyoxal, glyoxal and 3-deoxyglucosone are measured in a single run. Figure taken from Scheijen et al. (Clin Chem Lab Med, 2014).

Liquid chromatography (LC) has considerable advantages over ELISA based techniques. Firstly, and by far the most important advantage is that it allows the specific measurement of different AGEs, often in a single run. As the reader of this thesis will come to appreciate, the results differ considerably for the specific AGEs, underlining that the results of previous ELISA-based studies should be interpreted cautiously. Secondly, LC in general can measure most AGEs accurately and precisely even in very low concentrations. This is important for human studies, to overcome the large variation between people which may introduce confounding and weaken the

associations between AGEs and the outcomes of interest. Thirdly, LC requires only very low sample volumes, which is attractive for large cohort studies, where stored plasma samples are precious.

In this thesis, a new method to measure low concentrations of D-lactate in plasma and urine was developed (**Chapter 3**). In addition, this thesis reports on a refinement of a pre-existing AGE measurement, allowing the measurement of not only the protein-bound but also the free fraction of circulating AGEs (specifically: CML, CEL and MG-H1) (**Chapter 4**).

Although previous studies have investigated whether protein-bound plasma AGEs (CML, CEL and pentosidine) are associated with CVD in individuals with type 1 diabetes,⁵⁴ it is unknown whether these plasma AGEs are also associated with the presence of T2DM, and with CVD in individuals with T2DM. This is not a redundant question, since adipose tissue has been shown to trap AGEs (CML at least),⁵⁵ and since type 2 diabetes is usually characterized by concomitant adiposity, associations between plasma AGEs (CML particularly) and T2DM (**Chapter 4**) as well as CVD (**Chapter 6**) may be much weaker than those described for type 1 diabetes. Since it is known that AGEs not only accumulate in plaques but also in other organs key in developing T2DM, such as the liver,⁵⁶ and adipose tissue,⁵⁵ we measured AGEs directly in atherosclerotic plaques, to investigate whether the results obtained from plasma AGE measurements are similar to accumulation of AGEs in the atherosclerotic plaque (**Chapter 7**).

Knowledge gaps

Many unresolved questions remain regarding the extent to which MGO and AGEs contribute to progression of atherosclerotic plaques towards plaque rupture, and particularly human studies on this subject are lacking. Little research has focused on which specific circulating AGEs best reflect AGE accumulation in atherosclerotic lesions. The associations of specific plasma AGEs measured with LC and CVD have not been investigated in T2DM. Although previous studies suggest MGO, its derived AGEs and decreased GLO1 expression and/or activity may contribute to the increased risk of plaque rupture in inflammatory and metabolically active plaques, several important issues remain. A better understanding of the role AGE play in human atherosclerosis is needed to address whether AGEs hold promise to either improve the prediction or the treatment of CVD. A few of these major knowledge gaps will be addressed in this thesis:

1. It is unknown whether the ABI is as suitable a marker for CVD in individuals with diabetes, as it is in individuals without diabetes (**Chapter 2**).
2. No marker of the glyoxalase pathway suitable for use in large cohort studies exists. To this end, a sensitive assay to measure D-lactate was developed (**Chapter 3**).
3. Large cohort studies are needed to resolve whether plasma AGEs really are higher in T2DM (as they are in type 1 diabetes), independently of confounding factors. It is also unknown whether these associations differ for protein-bound or free plasma AGEs (**Chapter 4**).
4. Associations between circulating markers of the glyoxalase pathway, T2DM and prevalent CVD have not yet been investigated (**Chapter 5**).
5. Large cohort studies are needed to investigate whether plasma AGEs are associated with incident CVD in T2DM, independently of confounding factors (**Chapter 6**).
6. AGEs have been shown to be present in atherosclerotic plaques, but these studies have not supplied quantitative data, linking AGE levels to the phenotype of the plaque (**Chapter 7**).
7. GLO1 is considered a major line of defense against glycation and diabetic complications, but its expression has not been studied in relation to the atherosclerotic plaque phenotype (**Chapter 7**).
8. Hyperglycaemia has been identified as a cause for AGE formation in endothelial cells, but for macrophages, the most metabolically active cell of the plaque, the triggers for AGE formation are largely unknown (**Chapter 7**).
9. Overexpressing GLO1 has been shown to reduce AGEs in rats with diabetes, and prevent diabetic complications, but it is unknown if GLO1 overexpression is also protective against atherosclerosis (**Chapter 8**).

Thesis overview

Epidemiological studies strongly rely on the validity of the markers they use to reflect the concepts they investigate. To study the association between plasma AGEs, circulating markers of the glyoxalase pathway and T2DM and CVD, some remaining methodological issues were addressed. **Chapter 2** reports on the associations between the ABI and (cardiovascular) mortality in individuals with- and without diabetes (*knowledge gap 1*). **Chapter 3** describes a new method based on ultra performance tandem mass spectrometry to measure D-lactate in plasma and urine, as markers for the glyoxalase pathway (*knowledge gap 2*). In **Chapter 4** it is investigated whether plasma levels of AGEs are associated with having diabetes (*knowledge gap 3*) and prevalent CVD. This chapter also reports on the further refinement of a previous method to measure AGEs with LC, allowing measurement of both protein-bound and free AGEs. **Chapter 5** reports on associations between circulating GLO1 mRNA levels,

as well as plasma and urinary D-lactate levels and cIMT, ABI and prevalent CVD (*knowledge gap 4*). Next, associations between plasma AGEs and incident CVD in individuals with T2DM were studied in **Chapter 6** (*knowledge gap 5*). In **Chapter 7** it was investigated whether AGEs are indeed higher in rupture-prone plaques than in stable plaques (*knowledge gap 6*). In addition, GLO1 expression in ruptured plaques was measured in ruptured plaques (*knowledge gap 7*). Next, metabolic factors were determined that contribute to AGE and MGO accumulation and reduced GLO1 activity *in vitro* (*knowledge gap 8*). Furthermore, a mechanism through which MGO, GLO1 and AGEs link high metabolic activity in inflammatory plaques to development of a necrotic core was investigated using cell culture models. In **Chapter 8** it was investigated in mice whether GLO1 indeed protects against glycation in atherosclerosis (*knowledge gap 9*), and subsequently, against development of a phenotype resembling the human rupture-prone plaque. To this end, GLO1 overexpressing mice were crossed with ApoE deficient mice, a mouse model that due to a genetic modification spontaneously develops atherosclerosis over time. The results from this thesis are summarized and discussed in **Chapter 9**.

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Chapter 2

Associations between the ankle-brachial index and cardiovascular and all-cause mortality are similar in individuals without and with type 2 diabetes – 19-year follow-up of a population-based cohort study

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Abstract

Objective

In the general population, a low ankle-brachial index ($ABI < 0.9$) is strongly associated with (cardiovascular) mortality. However, the association between the ABI and mortality may be weaker in individuals with diabetes, as ankle pressures may be elevated by medial arterial calcification and arterial stiffening, which occur more frequently in diabetes. Therefore, the aim of this study was to compare the association between the ABI and mortality in individuals without and with diabetes.

Research design and methods

We studied the associations between the ABI and cardiovascular and all-cause mortality in 624 individuals from the Hoorn study, a population-based cohort of 50–75 years old individuals (155 with diabetes and 469 without) followed for a median period of 17.2 years. Data were analysed using Cox proportional hazard models.

Results

During the follow-up period, 289/624 (46.3%) participants died (97/155 with and 192/469 without diabetes; and 52/65 with and 237/559 without $ABI < 0.9$), 85 (29.4%) of CVD (30/155 with and 55/469 without diabetes; and 20/65 with and 65/559 without $ABI < 0.9$). A low ABI was strongly associated with cardiovascular mortality (RR: 2.57 (95% CI: 1.50 to 4.40)) and all-cause mortality (RR: 2.02 (95% CI: 1.47 to 2.76)), after adjustment for Framingham risk factors. The associations of the ABI with mortality did not differ between individuals without and with diabetes for cardiovascular ($p_{\text{interaction}} = 0.45$) or all-cause mortality ($p_{\text{interaction}} = 0.63$).

Conclusions

In the Hoorn Study, associations between the ankle-brachial index and cardiovascular and all-cause mortality were similar in individuals without and with diabetes. Future studies should investigate, in both individuals without and with diabetes, whether measurement of the ABI can be used to guide treatment decisions.

Introduction

Identifying individuals with a high risk of cardiovascular morbidity and mortality remains challenging, as many high-risk individuals are asymptomatic.¹ The ankle-brachial blood pressure index (ABI) is a marker for systemic atherosclerosis, is strongly associated with mortality,² and may thus improve identification of high-risk individuals. However, the validity of the ABI may be decreased in diabetes, as ankle pressures may be elevated by medial arterial calcification and arterial stiffening, which occur more frequently in diabetes.³ In diabetes, the ABI may thus fall within the normal range (≥ 0.9 to ≤ 1.4) when both atherosclerosis and arterial stiffening and calcifications of the lower limbs occur within the same individual. Therefore, the aim of this study was to investigate whether the associations between the ABI and cardiovascular and all-cause mortality are similar or, in fact, weaker in individuals with diabetes than in individuals without diabetes. We investigated the ABI at several cut-off points ($ABI < 0.9$, < 1.0 and < 1.1) and as a continuous variable, because development of atherosclerosis in the lower limbs is a continuous process.⁴ In addition, we investigated whether alternatives for the ABI, such as the toe brachial index (TBI) and Doppler flow curves of the lower extremities were more strongly associated with mortality than the ABI in individuals with diabetes, as these techniques may be less affected by arterial calcifications and stiffening than the ABI.

Research design and methods

We used data from the Hoorn Study, a population-based cohort study on glucose metabolism and other cardiovascular risk factors in a general Caucasian population, which has been described in detail previously.⁵ The Hoorn Study was approved by the ethical review committee of VU University Medical Centre, Amsterdam, the Netherlands. Informed consent was obtained from all participants, and all study participants gave written informed consent to be included. In brief, men and women aged 50-75 years were randomly selected from the population register of the town of Hoorn, the Netherlands; 2,484 subjects participated (response rate 71%). Baseline examinations were conducted from October 1989 until February 1992. All subjects had a 75-g oral glucose tolerance test, except those in whom type 2 diabetes had previously been diagnosed. An extensive metabolic and cardiovascular investigation was performed in an age-, sex-, and glucose-tolerance-stratified, random subsample of 631 participants (89% of those invited), which was used in the present study.⁵ Participants without data on the ABI or Framingham risk factors were excluded ($n=7$). The present study, therefore, consisted of 624 individuals: 371 with normal glucose metabolism, 98 with impaired glucose metabolism (including those with impaired fasting glucose and/or impaired glucose tolerance), and 155 with type 2 diabetes.⁶

Baseline measurements

Main determinants

The ABI was measured at baseline as described earlier in detail.⁵ In short, Doppler-assisted systolic blood pressure measurements were taken from the brachial and posterior tibial arteries on both sides, using 12-cm cuffs (Medasonics Vasculab, Mountainview, Calif., USA). Recording started after a 15 minute resting period in a supine position (room temperature 23°C). Whenever the ABI over the posterior tibial artery was less than 0.9, or the Doppler flow signal was not audible, the ankle pressure was also measured over the dorsalis pedis artery or the peroneal artery. The ABI was calculated for each leg using the highest ankle pressure divided by the highest systolic brachial pressure. An ABI of <0.9 in either leg was considered indicative of systemic atherosclerosis. In additional analyses, the associations between the ABI and mortality were reanalyzed with higher cut-off points (<1.1 and <1.0) and with the ABI as a continuous variable.

In diabetic individuals, the ABI may increase due to arterial stiffening and calcification of large vessels. Thus, in diabetic individuals methods alternative to the ABI, which may be less affected by these phenomena, may be more strongly associated with mortality than the ABI. We therefore measured toe pressures to calculate a toe brachial index (TBI), which may give a more valid impression of flow impairment in diabetes, as smaller vessels are thought to be less affected by arterial calcifications than larger vessels. The TBI was calculated for each leg by dividing the toe pressure by the highest brachial pressure. For the TBI a ratio of <0.7 is considered abnormal.⁷ In addition, we measured Doppler flow curves of the lower arteries, as even in calcified vessels atherosclerosis may yield abnormal flow patterns. With the use of a 5 or 8 mHz bi-directional continuous wave Doppler connected to a frequency analyzer, Doppler flow curves were determined at the left and right leg, at the level of the aorta-iliac, femoral-popliteal, dorsalis pedis, peroneal and tibial arteries.⁵ Triphasic or biphasic curves were considered normal, whereas a monophasic curve or the absence of a curve in one or more of the arterial tracts was considered abnormal.

Co-variables

Urinary albumin concentration was measured in a first-voided sample by rate nephelometry (Array Protein System, Beckman Coulter, Galway, Ireland) with a detection threshold of 6.2 mg/l (intra- and interassay coefficients of variation of 5 and 8%, respectively). Urinary creatinine was measured with a modified Jaffé method. Microalbuminuria was present if the albumin-to-creatinine ratio was in the range of 2.0–30 mg/mmol. Macroalbuminuria was present if the albumin-to-creatinine ratio was >30 mg/mmol. Body mass index (BMI); waist and hip circumference; systolic (SBP) and diastolic blood pressure (DBP); levels of fasting plasma glucose; HbA1c; insulin; total, high density (HDL), and low density (LDL) cholesterol; triglycerides and plasma

creatinine; and smoking status were measured as described elsewhere.⁵ Glomerular filtration rate (eGFR) was estimated by the short Modification of Diet in Renal Disease equation.⁸ Hypertension was defined as a SBP $\geq$ 140 mmHg and/or a DBP $\geq$ 90 mmHg and/or the use of antihypertensive drugs. Prior cardiovascular disease (CVD) was defined when individuals had any of the following: a history of myocardial infarction, stroke or transient ischaemic attack, abnormalities on a resting electrocardiogram (Minnesota codes 1.1-1.3, 4.1-4.3, 5.1-5.3, or 7.1), intermittent claudication, non-traumatic amputation, previous coronary bypass surgery or angioplasty.

Follow-up

Data on the participants' vital status up to 1 January 2009 were collected from the mortality register of the municipality of Hoorn. Information on cause of death was extracted from the medical records of the general practitioners and the local hospital and coded according to the ICD-9. Cardiovascular mortality, including sudden death, was defined by ICD-9 codes 390-459 and 798. Information on cause of death could not be obtained for 32 of the deceased individuals. All subjects were followed until death or end of follow-up, at which time they were censored.

Statistical analyses

Baseline characteristics between survivors and non-survivors were compared with the use of Student's *t* or χ^2 tests. Logistic regression models were used to investigate the associations between cardiovascular risk factors and having an abnormal ABI, TBI or Doppler flow curve. Cox proportional hazards regression models were used to calculate the crude and adjusted relative risks (RRs) and respective 95% CIs of cardiovascular and all-cause mortality associated with having, respectively, an abnormal ABI, TBI or Doppler flow curve.

To investigate whether the associations between ABI, TBI and Doppler flow curves and mortality were different between individuals without and with diabetes, interaction terms (presence of diabetes $\times$ presence of an abnormal ABI, TBI or Doppler flow curve respectively) were used. In addition, analyses stratified for the presence of diabetes were performed. When dummy variables for impaired glucose metabolism and diabetes were added to the model, the relative risks for all-cause and cardiovascular mortality of individuals with impaired glucose tolerance did not differ significantly from individuals with normal glucose tolerance. Therefore, individuals with impaired glucose metabolism were combined with individuals with normal glucose metabolism and compared with individuals with diabetes. To prevent overadjustment for peripheral arterial disease (PAD), self reported PAD ($n=1$) and non-traumatic amputation ($n=2$) were excluded from the definition of prior CVD when the associations between ABI, TBI and Doppler flow curves and mortality were adjusted for prior CVD.

For all analyses a two-sided p-value of <0.05 was considered statistically significant, except for the interaction terms, for which a two-sided p-value of <0.10 was considered statistically significant. All analyses were performed with SPSS (version 17.0).

Results

Median duration of follow-up was 17.2 years (range 0.5 to 19.2). During the follow-up period, 289/624 (46.3%) participants died (97/155 with and 192/469 without diabetes; and 52/65 with and 237/559 without $ABI < 0.9$), 85 (29.4%) of CVD (30/155 with and 55/469 without diabetes; and 20/65 with and 65/559 without $ABI < 0.9$). At baseline, individuals who died, as compared to those who survived, more often had an ABI of <0.9 , a TBI of <0.7 , and abnormal Doppler flow curves. In addition, male sex, type 2 diabetes, hypertension, lower eGFR, (micro)albuminuria, lower HDL-cholesterol and prior CVD occurred more frequently, and age, waist circumference and HbA1c were higher in the non-survivors as compared with the survivors (Table 2.1). Smoking, higher age, higher systolic blood pressure, lower HDL and prior CVD were independently associated with the ABI (data not shown). An ABI of <0.9 occurred more often in individuals with diabetes (14.8%) than in non-diabetic individuals (9.0%). In this cohort an ABI of >1.4 did not occur.

ABI and cardiovascular and all-cause mortality

A low ABI (<0.9) was strongly associated with cardiovascular mortality (RR: 2.57 (95% CI: 1.50 to 4.40)) and all-cause mortality (RR: 2.02 (95% CI: 1.47 to 2.76)), independently of the Framingham risk factors (Table 2.2, model 2). Additional adjustment for (micro)albuminuria, eGFR, triglycerides and prior CVD attenuated the association with cardiovascular mortality (RR: 1.83 (95% CI: 1.01 to 3.34)) and somewhat with all-cause mortality (RR: 1.79 (95% CI: 1.27 to 2.52)) (Table 2.2, model 3). The associations of the ABI (<0.9) with mortality did not differ between individuals without and with diabetes for cardiovascular ($p_{\text{interaction}}=0.45$) or all-cause mortality ($p_{\text{interaction}}=0.63$). When <1.1 was selected as a cut-off point for the ABI, the associations of the ABI with cardiovascular mortality ($p_{\text{interaction}}=0.04$) and all-cause mortality ($p_{\text{interaction}}=0.14$) were weaker in individuals with diabetes than in individuals without diabetes (Table 2.3). This was not the case when <1.0 was selected as a cut-off point for the ABI (both $p_{\text{interaction}} \geq 0.43$), or when the ABI was analyzed as a continuous variable (both $p_{\text{interaction}} \geq 0.54$) (Table 2.3).

Table 2.1 Baseline characteristics according to survival status after a median follow-up of 17.2 years (n=624)

	Survivors	Non-survivors	
		All-cause mortality	Cardiovascular mortality
N	335	289	85
Men (%)	44.2	52.6*	51.8*
Age (years)	61.7 ± 6.7	67.4 ± 6.5*	68.3 ± 6.5
Glucose metabolism status			
NGM (%)	68.4	49.1	47.1
IGM (%)	14.3	17.3	17.6
Type 2 diabetes (%)	17.3	33.6*	35.3*
HbA1c (%)	5.7 ± 1.0	6.2 ± 1.6*	6.2 ± 1.3*
SBP (mm Hg)	135.2 ± 18.2	144.0 ± 20.1*	146.3 ± 21.1*
DBP (mm Hg)	82.1 ± 9.1	83.1 ± 11.2	83.6 ± 11.9
Use of blood pressure-lowering drugs (%)	21.2	36.0*	56.5*
Hypertension (%)	30.1	49.5*	62.4*
Prior CVD (%)	19.4	27.7*	36.5*
Ever smokers (%)	57.0	68.9*	69.4*
BMI (kg/m ²)	26.9 ± 3.5	27.7 ± 4.4*	27.4 ± 4.5
Waist (men)	95.6 ± 8.5	98.8 ± 10.8*	98.1 ± 8.8
Waist (women)	88.4 ± 10.8	93.8 ± 12.0*	92.4 ± 13.0*
WHR	0.90 ± 0.08	0.94 ± 0.08*	0.94 ± 0.08*
Serum creatinine (umol/l)	89.2 ± 16.3	94.6 ± 21.4*	98.4 ± 29.8*
eGFR (ml/min/1.73m ²)	69.4 ± 10.8	65.8 ± 13.0*	63.6 ± 14.1*
Cholesterol (mmol/l)	6.6 ± 1.1	6.6 ± 1.2	6.8 ± 1.3
HDL cholesterol (mmol/l)	1.3 ± 0.4	1.2 ± 0.4*	1.2 ± 0.3
LDL cholesterol (mmol/l)	4.5 ± 1.0	4.5 ± 1.1	4.7 ± 1.1
Triglycerides (mmol/l)	1.5 (1.1-2.1)	1.7 (1.2-2.3)*	1.7 (1.2-2.3)*
Use of lipid-lowering drugs (%)	1.8	1.4	1.2
Microalbuminuria (%)	6.0	17.2*	25.6*
Macroalbuminuria (%)	0.3	2.2*	3.7*
Ankle-brachial index <0.9 (%)	3.9	18.0*	23.5*
Ankle-brachial index >1.4 (%)	0	0	0
Toe-brachial index <0.7 (%)	16.6	26.8*	31.8*
Abnormal flow curve (%)	14.9	26.3	29.4*

Data are presented as frequencies (%), means ± SD, or medians (interquartile range). *P<0.05 vs survivors. NGM, normal glucose metabolism, IGM: impaired glucose metabolism, HbA1c: glycated hemoglobin, SBP: systolic blood pressure, DBP: diastolic blood pressure, CVD: cardiovascular disease, BMI: body mass index, WHR: waist-to-hip ratio, eGFR, estimated glomerular filtration rate

Alternative methods to the ABI and their associations with cardiovascular and all-cause mortality

A low TBI was positively associated with cardiovascular mortality (RR: 1.30 (95% CI: 0.77 to 2.20)), and with all-cause mortality (RR: 1.16 (95% CI: 0.86 to 1.56)). Abnormal Doppler flow curves were also positively associated with cardiovascular mortality (RR: 1.27 (95% CI: 0.74 to 2.18)) and with all-cause mortality (RR: 1.37 (95% CI: 1.02 to 1.84)). However, as compared with the ABI, the TBI and Doppler flow curves were not

more strongly associated with all-cause and cardiovascular mortality than the ABI (Table 2.2). This was still the case if we stratified the above associations for the presence of diabetes (data not shown).

Table 2.2 Associations of ABI, TBI or abnormal Doppler flow curves with cardiovascular and all-cause mortality.

	Model*	All-cause mortality		Cardiovascular mortality	
		RR	95% CI	RR	95% CI
ABI	1	2.18	1.60 to 2.97	3.02	1.80 to 5.05
	2	2.02	1.47 to 2.76	2.57	1.50 to 4.40
	3	1.79	1.27 to 2.52	1.83	1.01 to 3.34
TBI	1	1.31	1.00 to 1.71	1.67	1.04 to 2.66
	2	1.24	0.94 to 1.62	1.51	0.93 to 2.46
	3	1.16	0.86 to 1.56	1.30	0.77 to 2.20
Doppler flow curves	1	1.44	1.10 to 1.88	1.50	1.19 to 1.91
	2	1.36	1.04 to 1.78	1.50	0.93 to 2.43
	3	1.37	1.02 to 1.84	1.27	0.74 to 2.18

Data were analyzed using Cox regression analyses. *Model 1: adjusted for age, sex and glucose metabolism status; model 2: Model 1 plus adjustment for other Framingham risk factors (total cholesterol, HDL-cholesterol, smoking, systolic blood pressure); model 3: model 2 plus prior CVD, triglycerides, (micro)albuminuria, eGFR and waist circumference.

Table 2.3 Associations of the ABI at different cut-off points and as a continuous variable with all-cause and cardiovascular mortality.

	Cut-off point	All-cause mortality		Cardiovascular mortality	
		RR	95% CI	RR	95% CI
All individuals	Continuous	1.13	1.07 to 1.19	1.09	0.99 to 1.20
	<1.1	1.33	0.97 to 1.83	1.07	0.60 to 1.91
	<1.0	1.30	0.99 to 1.70	1.32	0.80 to 2.18
	<0.9	1.79	1.27 to 2.52	1.83	1.01 to 3.34
Non-diabetic individuals	Continuous	1.10	1.03 to 1.18	1.08	0.96 to 1.23
	<1.1	1.48	1.00 to 2.18	1.55	0.73 to 3.26
	<1.0	1.22	0.87 to 1.71	1.39	0.75 to 2.61
	<0.9	1.84	1.18 to 2.88	1.95	0.88 to 4.33
Diabetic individuals	Continuous	1.15	1.05 to 1.27	1.06	0.89 to 1.26
	<1.1	1.11	0.61 to 2.01	0.62	0.21 to 1.80
	<1.0	1.52	0.94 to 2.44	1.44	0.58 to 3.54
	<0.9	1.74	0.97 to 3.14	1.30	0.46 to 3.62

Data were analyzed using Cox regression analyses. For the ABI as a continuous variable, the RR is expressed per 0.1 decrease in ABI. All analyses were adjusted for sex, age, smoking, total and HDL-cholesterol, triglycerides, albuminuria, eGFR, waist circumference and prior CVD. In the whole cohort all analyses were adjusted for glucose metabolism status, in the stratified analysis individuals with impaired glucose tolerance were combined with individuals with normal glucose metabolism and compared with individuals with diabetes. Analyses in individuals without diabetes were therefore additionally adjusted for the presence of impaired glucose metabolism.

Discussion

This study had two main findings. Firstly, a low ABI was independently associated with cardiovascular and all-cause mortality in individuals without and with diabetes. Secondly, in individuals with diabetes, the TBI and Doppler flow curves were not superior to the ABI, i.e. were not more strongly related to mortality than the ABI.

In the MERITO II study,⁹ the association between a low ABI and cardiovascular mortality was significantly weaker in individuals with diabetes than in individuals without diabetes. However, that study had a follow-up duration of only 1 year, which may explain the difference with the current study. On the other hand, in the PAMISCA study, in individuals with acute myocardial infarction, the association between PAD and all-cause mortality at 1-year follow-up was not weaker in individuals without compared to those with diabetes.¹⁰ Although in this study individuals with a low ABI and symptomatic peripheral arterial disease (such as revascularisation procedures or ischaemic amputations) were analysed as a single group, it is unlikely that this changed the associations between ABI and mortality in this study, as of the 421 individuals with PAD, only 30 had such symptomatic disease. Also in line with our findings, in the Strong Heart Study, and the 'men born in 1914' study, the association between a low ABI and mortality was similar in individuals without and with diabetes at longer follow-up times.^{11,12} Taken together, these studies support our finding that the association between the ABI and mortality does not differ between individuals without and with diabetes, especially at long-term follow-up.

Although the TBI and Doppler flow curves have been advocated as alternatives to the ABI in individuals with an elevated (>1.4) ABI,⁷ and the TBI has been shown to predict mortality in individuals with an elevated ABI,¹³ we show here that in a population without elevated ABI values the TBI and Doppler flow curves are not more strongly associated with mortality than the ABI, both in individuals without and with diabetes. In addition, and in line with the 'men born in 1914' study,¹¹ we show that individuals with elevated ABI values are uncommon in the general Caucasian population. Thus, the current study suggests that both the TBI and Doppler flow curves are not more strongly associated with mortality than the ABI in individuals with diabetes and an ABI in the non-elevated range, but additional studies are needed to confirm this.

This study was done in a middle-aged to elderly Caucasian population, and thus its results may not apply to other populations. In addition, due to the trend of increasing obesity in the Western world during the follow-up of our study, it may be argued that the participants in our study are perhaps at baseline less representative in terms of BMI of the patients with diabetes currently seen in clinical practice. However, there is no biological reason why this should affect the association between ABI and cardiovascular outcomes. In addition, our study lacked power to perform analyses to investigate whether the ABI improves risk prediction of mortality. The advantage of

our study over the MERITO II and PAMISCA study is its longer follow-up time.^{9,10} Compared to the Strong Heart Study, our study adds data on the performance of the ABI in an older, Caucasian population, and to 'the men born in 1914' study the inclusion of women and a larger variance in age categories.

Taken together, our data show that the ABI is a suitable tool to investigate the association between systemic atherosclerosis and mortality in individuals with diabetes, as it is in individuals without diabetes.² This study underlines that, at its conventional cut-off point of <0.9 , the association between ABI and all-cause and cardiovascular mortality does not differ between individuals without and with diabetes. We did find an interaction between the ABI and diabetes at a cut-off point of 1.1. Although this finding may be due to chance, as we did not find an interaction between the ABI and diabetes when we analysed the ABI at lower cut-off points (<1.0 and <0.9) or as a continuous variable, this finding may indicate that arterial stiffening and calcification in diabetes may elevate the ABI only in the normal to high range (>1.0), but not in the lower ranges (0 to 1.0), and is not of clinical importance at the regular cut-off point of the ABI (<0.9). In addition, alternatives to the ABI are not more strongly associated with mortality than the ABI in individuals with diabetes.

The ABI may be an important tool to improve risk prediction. In line with this, the ACCF/AHA 2010 guideline states that measurement of the ABI in individuals at intermediate risk is reasonable to improve cardiovascular risk prediction.¹⁴ In addition, the ADA recommends measurement of the ABI in individuals with diabetes to help prevent complications of peripheral arterial disease, and identify patients at high cardiovascular risk.¹⁵ However, there are currently no studies that investigated whether measurement of the ABI is actually effective in motivating patients to comply with measures to reduce cardiovascular risk, or that serial measurement of the ABI can be used to monitor or guide treatment approaches,¹⁴ and future studies should investigate this.

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Chapter 3

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 0.65 $\mu\text{mol/l}$ and 0.2 $\mu\text{mol/l}$ 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 3.1B). 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 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-lactate in controls and in 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 ($\geq 99.9\%$) 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 ($\geq 97\%$) (DATAN) and lithium D-lactate ($\geq 99\%$) were obtained from Fluka. Water and acetonitril (ULC-MS grade) were obtained from Biosolve. [$^{13}\text{C}_3$]-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 C_{18} 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 $\mu\text{mol/l}$), [$^{13}\text{C}_3$]-L-lactate (400 $\mu\text{mol/l}$) and L-lactate (1000 $\mu\text{mol/l}$) standard solution into the tandem MS at a flow of 20 $\mu\text{l/min}$. 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 3.1 shows the optimised MRM settings.

Table 3.1 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 $5.6 \pm 0.1\%$ and a fasting plasma glucose of 5.2 ± 0.1 mmol/l. Group 2, the patients with diabetes ($n=52$), were 56.3 ± 0.6 years old, 39% female and had a HbA1C of $6.9 \pm 0.2\%$ 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 , 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 μ l of internal standard solution (containing 434.75 μ mol/l [¹³C₃]-L-lactate) 25 μ l of plasma was added. Samples were mixed thoroughly and subsequently deproteinized with 600 μ l 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 μ l acetonitril:water (1:2, by volume).

Urine sample preparation

Twenty-five microliters of internal standard solution (containing 434.75 $\mu\text{mol/l}$ [$^{13}\text{C}_3$]-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 3.2). 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, [$^{13}\text{C}_3$]-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 samples in liquid nitrogen and thawing and this 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.

Table 3.2 Linearity tested in different matrices.

Matrix	Slope ^(a)	Y-intercept	r ²	Concentration range (μmol/l)
D-lactate				
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)

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.³⁰

D-lactate enzymatic assay

For method comparison, an enzymatic-spectrophotometric method, based on the oxidation of D-lactate to 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 3.1.

Stability of D- and L-lactate

After 5 freeze-thaw cycles no significant change of D- and L-lactate levels was observed in both plasma (plasma A: D-lactate 10.6 μM (7.8%), L-lactate 1290 μM (2.1%); plasma B: D-lactate 70.4 μM (5.5%), L-lactate 6439 μM (3.7%); mean (CV%)) and urine samples (urine A: D-lactate 119.6 μM (13.1%), L-lactate 112.6 μM (4.9%); urine B: D-lactate 17.3 μM (2.3%), L-lactate 66.5 μM (2.2%); mean (CV%)).

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 plasma and urine samples (plasma A: D-lactate 11.6 μM (11.7%), L-lactate 1553 μM (3.5%); plasma B: D-lactate 75.3 μM (3.1%), L-lactate 6561 μM (4.9%); mean (CV%)) and urine samples (urine A: D-lactate 127.2 μM (1.9%), L-lactate 115.5 μM (1.4%); urine B: D-lactate 18.3 μM (3.9%), L-lactate 65.0 μM (1.2%); mean (CV%)).

Figure 3.1 Representative chromatograms of D- and L-lactic acid derivatives ^(a)
A: Internal standard $[^{13}\text{C}_3]$ -L-lactate chromatogram (435 $\mu\text{mol/l}$, 72 pmol). **B:** Representative chromatogram of a standard solution of D- and L-lactate (351 $\mu\text{mol/l}$, 58.2 pmol and 501 $\mu\text{mol/l}$, 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 $\mu\text{mol/l}$, 2.3 pmol and 74.6 $\mu\text{mol/l}$, 12.4 pmol respectively). **D:** Representative chromatogram of a plasma sample (D- and L-lactate; 11.2 $\mu\text{mol/l}$, 1.9 pmol and 1375.0 $\mu\text{mol/l}$, 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 $1.4 \pm 6.3\%$ (mean $\pm$ CV%) for D-lactate and $0.75 \pm 3.2\%$ for L-lactate (Table 3.2). For urine the slope, tested in three different urine samples and in water measured on different days, was $1.09 \pm 4.0\%$ for D-lactate and $0.80 \pm 1.6\%$ for L-lactate (Table 3.2). The limits of detection of D-lactate and L-lactate in plasma were 0.65 $\mu\text{mol/l}$ (108 fmol) and 0.2 $\mu\text{mol/l}$ (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 3.3). The validation data demonstrated inter- and intra-assay variations between 2% and 9% (Table 3.4).

Table 3.3 Recovery data for D- and L-lactate in 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) μmol/l (n=5)			
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) μmol/l (n=5)			
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

Addition of 25 µl standard solution to 25 µl plasma or urine. ^(b) Creatinine concentration: 15.4 mmol/l.

Table 3.4 Precision data D- and L-lactate in plasma and urine.

Matrix	D-lactate	CV,%	L-lactate	CV,%
Intra-assay, n=10	Mean (SD), µmol/l		Mean (SD), µmol/l	
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	
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 D-lactate levels in plasma and 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 3.2A). However, the Bland Altman plot showed that although in the

higher range both techniques are in excellent agreement, in the lower range considerably higher values were measured with the enzymatic technique as compared with the UPLC tandem MS method (Figure 3.2B).

Figure 3.2 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 concentrations of non-diabetic controls, patients with T2DM and patients with IBD (Figure 3.3). In healthy controls, D- and L-lactate concentrations were 8.0 ± 0.6 and 1044.8 ± 36.7 $\mu\text{mol/l}$ in plasma, respectively, and 1.1 ± 0.2 and 6.3 ± 0.9 $\mu\text{mol/mmol}$ creatinine in urine, respectively (mean $\pm$ SEM). For T2DM patients the concentrations of D- and L-lactate were higher in plasma (12.3 ± 0.8 and 1534.7 ± 67.5 $\mu\text{mol/l}$, respectively) and urine (3.4 ± 1.0 and 12.1 ± 2.0 $\mu\text{mol/mmol}$ creatinine, respectively). For L-lactate this was

significant. 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).

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 $\mu\text{mol/l}$, respectively) and urine (3.1 ± 0.8 and 11.8 ± 1.4 $\mu\text{mol/mmol creatinine}$, respectively). This was significant for urinary L-lactate.

Figure 3.3 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 $\pm$ 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, this was 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.^{3,12,13,31} Furthermore, the shorter lifetime, higher cost and difficult selection of a suitable chiral column^{32,33} 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 synthesised before use.

Anhydrides of tartaric acid were used successfully as chiral derivatisation reagents of hydroxy acids and other enantiomeric compounds.³⁴⁻³⁶ 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. Other authors report a significant increase

of D-lactate in hospitalised patients with active IBD.⁶ This may be explained by the fact that the patients we have included were in remission. In addition, due to the relatively small sample size, we may lack power to detect a statistically significant difference in this group, as the levels of D-lactate in the IBD group were comparable to the levels observed in the T2DM group.

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.³⁷ 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 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 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.

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Chapter 4

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 positive²⁻⁷ 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, 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.

Study population

The present study included participants from the baseline examination of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) Study¹¹ and from the follow-up examination of the Hoorn Study,¹² 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 the online-only supplement.

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.^{11,12} 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.^{11,12} 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 $2 \times \text{DBP} + \text{SBP} / 3$), total, HDL and LDL cholesterol, triglycerides, estimated (e) GFR (using the short Modification of Diet in Renal Disease equation¹³), presence of micro- or macroalbuminuria and use of BP-, lipid- and glucose-lowering medication, and were determined as described earlier.^{11,12}

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 4.1.

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 4.1), after adjustment for potential confounders (Supplementary Table S4.1), 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 to 0.297)), an increase that was not clearly linear across deteriorating GMS (Supplementary Table S4.1).

Similar results were found when we investigated the linear associations of fasting glucose and HbA1c with plasma AGEs (Supplementary Table S4.2), or when we analysed the Hoorn and CODAM study separately (Supplementary Table S4.3).

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 4.1). However, after full adjustment for potential confounders (Table 4.2), 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 to 1.81)), but an inverse association between this AGE and CVD in individuals with T2DM (OR: 0.79 (95% CI 0.63 to 0.99)) (Table 4.2). We found similar results when we analysed the Hoorn and CODAM cohorts separately (Supplementary Table S4.4).

Table 4.1 General characteristics of the study population stratified according to prior CVD or glucose metabolism status.

	Prior CVD		P-value	Glucose metabolism status		
	No (n=733)	Yes (n=558)		NGM (n=573)	IGM (n=304)	T2DM (n=414)
Age (years)	63 ± 8	68 ± 8	<0.001	64 ± 8	66 ± 8	65 ± 8
Sex (% women)	45	45	0.911	46	45	45
NGM/IGM/T2DM (%)	50/23/27	37/24/39	<0.001	-	-	-
Prior CVD (%)	-	-	-	36	43	53
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
Waist circumference (cm)	97 ± 12	98 ± 12	0.046	94 ± 11	99 ± 11	102 ± 12
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
HbA1c (%)	5.9 ± 0.7	6.1 ± 0.8	<0.001	5.7 ± 0.4	5.9 ± 0.4	6.6 ± 1.0
Glucose-lowering medication (%)	9	10	0.236	0	1.0	28.0
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
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
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
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)
Lipid-lowering medication (%)	10	28	<0.001	14	17	23
Current smoking (%)	17	17	0.950	19	18	14
Systolic BP (mmHg)	138 ± 18	147 ± 20	<0.001	136 ± 19	145 ± 18	148 ± 19
Diastolic BP (mmHg)	79 ± 9	80 ± 9	0.338	77 ± 9	81 ± 9	81 ± 9
Mean BP (mmHg)	99 ± 11	102 ± 12	<0.001	97 ± 11	102 ± 11	104 ± 11
Hypertension ^a (%)	56	79	<0.001	53	71	81
BP-lowering medication (%)	28	53	<0.001	27	40	54
eGFR (ml/min/1.73m ²)	84 ± 17	81 ± 19	<0.001	82 ± 15	82 ± 17	85 ± 21
Albuminuria ^a (%)	93/6/1	85/14/1	<0.001	93/6/1	90/9/1	84/15/1
<i>Protein-bound AGEs</i>						
CML (nmol/mmol lysine)	34 (29-39)	33 (27-38)	0.004	34 (30-41)	33 (28-37)	31 (26-37)
CEL (nmol/mmol lysine)	28 (22-35)	32 (25-40)	<0.001	28 (23-35)	31 (23-38)	31 (22-39)
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)
<i>Free AGEs^c</i>						
CML (nmol/l)	77 (60-96)	83 (66-101)	0.070	78 (61-100)	77 (58-96)	80 (64-98)
CEL (nmol/l)	45 (36-56)	48 (39-62)	0.015	44 (36-55)	44 (36-56)	49 (39-64)
MG-H1 (nmol/l)	116 (84-165)	141 (96-209)	<0.001	127 (91-169)	115 (84-173)	121 (84-183)

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

Table 4.2 Associations of plasma protein-bound and free AGEs with prevalent CVD in the total population and stratified according to glucose metabolism status.

Independent variable	Model	Total population (n=1,1291)			NGM (n=573)			IGM (n=304)			T2DM (n=366)		
		OR	95% CI	P	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
Protein-bound													
Log _e CML	1	0.89	0.79 to 1.01	0.064	0.96	0.78 to 1.17	0.673	0.92	0.69 to 1.23	0.568	0.81	0.67 to 0.98	0.028
	2	0.97	0.85 to 1.12	0.711	1.04	0.82 to 1.33	0.726	1.15	0.82 to 1.62	0.430	0.85	0.68 to 1.06	0.142
Log _e CEL	1	1.04	0.93 to 1.17	0.505	1.00	0.83 to 1.22	0.973	1.37	1.07 to 1.76	0.013	0.90	0.74 to 1.10	0.313
	2	1.00	0.88 to 1.14	0.982	1.07	0.86 to 1.32	0.555	1.37	1.04 to 1.81	0.025	0.79	0.63 to 0.99	0.038
Log _e Pentosidine	1	1.04	0.92 to 1.17	0.587	1.22	0.99 to 1.49	0.059	0.83	0.62 to 1.12	0.218	0.98	0.82 to 1.18	0.983
	2	1.07	0.93 to 1.22	0.353	1.17	0.92 to 1.48	0.202	1.01	0.75 to 1.36	0.961	1.04	0.84 to 1.28	0.740
Free ^a													
Model		Total population (n=554 ^b)			NGM (n=287 ^a)			IGM (n=123 ^a)			T2DM (n=144 ^a)		
		OR	95% CI	P	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
Log _e CML	1	1.08	0.88 to 1.32	0.467	1.21	0.90 to 1.62	0.201	0.98	0.65 to 1.47	0.902	0.96	0.66 to 1.40	0.829
	2	0.96	0.76 to 1.21	0.709	1.09	0.78 to 1.53	0.603	1.00	0.79 to 1.28	0.998	0.63	0.37 to 1.08	0.095
Log _e CEL	1	1.12	0.92 to 1.36	0.277	1.30	0.97 to 1.73	0.079	0.94	0.60 to 1.47	0.770	1.00	0.71 to 1.42	0.996
	2	1.01	0.81 to 1.27	0.915	1.12	0.86 to 1.68	0.291	1.04	0.80 to 1.36	0.880	0.72	0.45 to 1.15	0.174
Log _e MG-H1	1	1.31	1.07 to 1.60	0.009	1.37	1.00 to 1.88	0.048	1.21	0.80 to 1.83	0.375	1.25	0.89 to 1.75	0.199
	2	1.21	0.96 to 1.53	0.101	1.29	0.90 to 1.86	0.175	1.24	0.97 to 1.58	0.377	1.08	0.69 to 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_r, 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 positive²⁻⁷ 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 4.1). 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,¹⁴ ageing,¹⁵ renal function¹⁶ or use of lipid-lowering medication¹⁷ 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 AGEs¹⁸ 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.¹⁹

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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Supplementary methods

Study population

The present study included participants from the baseline examination of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) Study¹ and from the follow-up examination of the Hoorn Study², 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 detail below.

Protein-bound AGEs

For measurement of protein-bound CML, CEL and lysine, 25 μl plasma was mixed with 50 μl water. After addition of 200 μl sodium borohydride (100 mmol/l) dissolved in borate buffer (pH 9.2, 200 mmol/l), samples were incubated at room temperature for 2 hours. Next, samples were deproteinized with 1000 μl cold (4°C) trifluoroacetic acid. After centrifugation (4300 g, 4°C , 20 min) the supernatant was carefully removed. Samples were then hydrolyzed by adding 500 μl 6 N HCl to the protein pellet and incubated for 18 hours at 110°C . After hydrolysis, 40 μl hydrolysate and 20 μl internal standard (containing 1432 nmol/l [$^2\text{H}_2$]-CML and 1378 nmol/l [$^2\text{H}_4$]-CEL (Polypeptide

Laboratories, San Diego, CA, USA) was mixed in a reaction vial. For CML and CEL, this mixture was evaporated to dryness under a stream of nitrogen at 70°C and subsequently derivatized with 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. For measurement of lysine, 10 µl hydrolysate was diluted in 800 µl water. Twenty µl of this mixture and 20 µl internal standard [$^{13}\text{C}_6$]-L-Lysine (40.16 µmol/l, Cambridge Isotope Laboratories, Andover, MA, USA) was diluted in 500 µl 10 mmol/l ammonia. Derivatized CML and CEL, and underivatized lysine were analyzed by UPLC (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 for CML and CEL, and a linear gradient of 20 mmol/l ammonium formate and acetonitril at 30°C for lysine. Flow rate was 800 µl/min and injection volume was 2 µl. Quantification of CML, CEL and lysine was performed by calculating the peak area ratio of each unlabeled peak area to the corresponding internal standard peak area. MRM transitions for CML, CEL and lysine were 317.1>186.1, 331.1>186.1 and 147.2>84.2, respectively. MRM transitions for the internal standards [$^2\text{H}_2$]-CML, [$^2\text{H}_4$]-CEL and [$^{13}\text{C}_6$]-L-Lysine were 319.1>186.1, 335.1>190.1 and 153.2>89.2, respectively. Electrospray ionization was done at a capillary voltage of 0.5 kV, source temperature of 150°C and desolvation temperature of 600°C. For validation purposes, linearity was determined by adding standard solution of CML, CEL and lysine to water and plasma. Six-point calibration curves were prepared for CML (0-5250 nmol/l), CEL (0-6250 nmol/l) and lysine (0-198.2 µmol/l). The peak area ratio of CML and CEL multiplied by the concentration of each corresponding internal standard were plotted as a function of the concentration. Calibration curves for CML, CEL and lysine were linear over the described concentration ranges ($r^2>0.99$) in both water and plasma. Mean slopes (response factors) for CML, CEL and lysine were 1.064 (CV, 8.9%), 1.429 (CV, 6.0%) and 0.994 (CV, 4.2%), respectively. Intra- and inter-assay coefficients of variation were 4.8 and 7.0% for CML, 5.0 and 9.7% for CEL, and 2.7 and 4.9% for lysine, respectively. The lower limits of quantification (s/N=10) on column were 4.0 fmol both for CML and CEL and 11 fmol for lysine, corresponding to a concentration of 200 nmol/l for CML and CEL and 254 µmol/l for lysine. Method correlation of the described lysine analysis was tested in 44 plasma samples as compared with a biconinonic acid (BCA) protein assay. The described lysine analysis gave good correlation with the protein analysis ($r^2=0.930$). Protein-bound pentosidine was measured using HPLC with a fluorescent detector, as described in detail elsewhere.³ Intra- and inter-assay CVs for pentosidine were 6.5% and 3.1%, respectively. Concentrations of protein-bound CML, CEL and pentosidine were adjusted for levels of lysine and expressed as nmol/mmol lysine.

Free AGEs

These measurements were confined to the CODAM Study population only, because plasma samples from the Hoorn Study were unavailable for this purpose. In short, to 25 μ l internal standard, 50 μ l plasma was added. Samples were mixed thoroughly and subsequently deproteinized with 600 μ l 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. Mean slopes (response factors) for CML, CEL and MG-H1 were 1.050 (CV, 10.1%), 1.460 (CV, 3.2%) and 1.010 (CV, 3.9%), respectively. Intra- and inter-assay CVs were 2.8 and 7.1% for CML, 3.7 and 6.4% for CEL, and 3.7 and 5.1% for MG-H1, respectively.

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 $2 \times \text{DBP} + \text{SBP} / 3$), total, HDL and LDL cholesterol, triglycerides, estimated (e) GFR (using the short Modification of Diet in Renal Disease equation⁴), 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 ($\geq 7.5\%$) HbA1c. Therefore, all results are shown without stratification for these factors.

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Table S4.1 Comparisons of levels of plasma protein-bound and free AGEs across levels of glucose metabolism.

Dependent variable	Model	IGM vs. NGM			T2DM vs. NGM			<i>P</i>	<i>P</i> _{linear trend}
		β	95% CI	<i>P</i>	β	95% CI	<i>P</i>		
<i>Protein-bound</i>									
Log _e CML	1	-0.299	-0.435 to -0.164	<0.001	-0.455	-0.579 to -0.331	<0.001	<0.001	
	2	-0.090	-0.219 to 0.040	0.175	-0.121	-0.262 to 0.020	0.093	0.078	
Log _e CEL	1	0.097	-0.042 to 0.236	0.173	-0.022	-0.149 to 0.105	0.735	0.829	
	2	0.055	-0.088 to 0.198	0.449	-0.090	-0.246 to 0.066	0.258	0.352	
Log _e Pentosidine	1	-0.155	-0.291 to -0.019	0.026	0.018	-0.106 to 0.142	0.772	0.922	
	2	-0.012	-0.145 to 0.121	0.861	0.152	0.007 to 0.297	0.040	0.060	
<i>Free^a</i>									
Log _e CML	1	-0.119	-0.323 to 0.085	0.253	-0.083	-0.287 to 0.112	0.404	0.329	
	2	-0.124	-0.326 to 0.077	0.228	-0.128	-0.380 to 0.124	0.321	0.223	
Log _e CEL	1	-0.061	-0.267 to 0.145	0.561	0.215	0.018 to 0.412	0.033	0.060	
	2	-0.099	-0.302 to 0.105	0.342	0.070	-0.183 to 0.323	0.588	0.866	
Log _e MG-H1	1	-0.168	-0.373 to 0.037	0.108	-0.098	-0.294 to 0.099	0.329	0.236	
	2	-0.171	-0.380 to 0.038	0.100	-0.134	-0.387 to 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. ^aRefers to data that are confined to the CODAM Study population.

Table S4.2 Associations of plasma AGEs with fasting glucose and HbA1c.

	Model	Fasting glucose		HbA1c	
		β	95%-CI	β	95%-CI
<i>Protein-bound</i>					
Log _e CML	1	-0.118	-0.173 to -0.063	-0.084	-0.140 to -0.027
	2	0.028	-0.020 to 0.077	0.051	-0.004 to 0.106
Log _e CEL	1	0.025	-0.030 to 0.080	0.040	-0.016 to 0.095
	2	0.018	-0.026 to 0.062	0.042	-0.007 to 0.091
Log _e Pentosidine	1	0.035	-0.021 to 0.091	0.030	-0.026 to 0.086
	2	0.050	0.002 to 0.097	0.051	-0.001 to 0.103
<i>Free^a</i>					
Log _e CML	1	-0.021	-0.107 to 0.064	0.031	-0.058 to 0.120
	2	-0.015	-0.077 to 0.048	0.039	-0.043 to 0.120
Log _e CEL	1	0.129	0.046 to 0.213	0.130	0.039 to 0.221
	2	0.046	-0.016 to 0.107	0.075	-0.011 to 0.160
Log _e MG-H1	1	-0.031	-0.116 to 0.055	0.016	-0.073 to 0.104
	2	-0.024	-0.086 to 0.037	0.018	-0.062 to 0.098

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. ^aRefers to data that are confined to the CODAM Study population.

Table S4.3 Comparisons of levels of plasma protein-bound AGEs across levels of glucose metabolism stratified according to cohort.

Dependent variable	Model	IGM vs. NGM			T2DM vs. NGM		
		β	95% CI	P	β	95% CI	P
<i>CODAM</i>							
Log _e CML	1	-0.250	-0.454 to -0.046	0.016	-0.638	-0.833 to -0.433	<0.001
	2	-0.085	-0.186 to 0.015	0.397	-0.336	-0.462 to -0.210	0.008
Log _e CEL	1	-0.087	-0.297 to 0.124	0.421	-0.285	-0.486 to -0.083	0.006
	2	-0.152	-0.263 to -0.042	0.150	-0.389	-0.628 to -0.149	0.005
Log _e Pentosidine	1	-0.197	-0.404 to 0.011	0.063	-0.013	-0.211 to 0.185	0.898
	2	-0.068	-0.263 to 0.127	0.503	0.041	-0.208 to 0.291	0.745
<i>Hoorn</i>							
Log _e CML	1	-0.325	-0.507 to -0.143	<0.001	-0.334	-0.496 to -0.172	<0.001
	2	-0.080	-0.251 to 0.091	0.359	-0.007	-0.182 to 0.168	0.937
Log _e CEL	1	0.253	0.069 to 0.436	0.007	0.201	0.038 to 0.365	0.016
	2	0.223	0.033 to 0.412	0.021	0.112	-0.083 to 0.306	0.260
Log _e Pentosidine	1	-0.121	-0.302 to 0.059	0.188	0.058	-0.103 to 0.219	0.481
	2	0.018	-0.161 to 0.196	0.845	0.207	0.024 to 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 S4.4 Associations of plasma AGEs with prevalent CVD stratified according to cohort.

Independent variable	Model	CVD		
		OR	95% CI	P
<i>CODAM</i>				
Log _e CML	1	0.77	0.62 to 0.95	0.013
	2	0.91	0.81 to 1.03	0.453
Log _e CEL	1	0.99	0.82 to 1.21	0.942
	2	0.95	0.85 to 1.06	0.654
Log _e Pentosidine	1	0.87	0.71 to 1.06	0.173
	2	0.94	0.75 to 1.18	0.597
<i>Hoorn</i>				
Log _e CML	1	0.96	0.82 to 1.12	0.576
	2	1.00	0.84 to 1.20	0.970
Log _e CEL	1	1.07	0.92 to 1.24	0.397
	2	1.03	0.88 to 1.22	0.697
Log _e Pentosidine	1	1.16	0.98 to 1.37	0.085
	2	1.17	0.98 to 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.

Chapter 5

Circulating glyoxalase 1 mRNA and plasma D-lactate levels are higher in type 2 diabetes, but are not associated with markers of atherosclerosis and presence of cardiovascular disease:

the CODAM study

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Abstract

Introduction

Advanced glycation endproducts (AGEs) contribute to the development of diabetic complications and cardiovascular disease (CVD). Methylglyoxal (MGO) has been identified as the most reactive AGE precursor. Glyoxalase 1 (GLO1) is the rate-limiting enzyme to convert MGO into D-lactate, and GLO1 overexpression has been shown to be protective against diabetic complications in rats. Although GLO1 was decreased in human ruptured plaques, it remains unclear whether general GLO1 expression is associated with type 2 diabetes (T2DM) and atherosclerotic disease in humans.

Methods

We measured levels of circulating GLO1 mRNA in whole blood of 474 individuals with normal glucose metabolism ($n=191$; women 37.7%; age 64.7 ± 7.3), impaired glucose metabolism ($n=116$; 42.28%; 66.9 ± 6.9) or T2DM ($n=167$; 37.1%; 67.5 ± 6.3) derived from the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) follow-up study. Plasma and urinary D-lactate levels were measured with UPLC tandem mass spectrometry.

Carotid intima media thickness (cIMT), ankle brachial index (ABI) and prevalent cardiovascular disease (CVD) were used as markers for atherosclerotic disease. GLO1 mRNA and plasma and urinary D-lactate were $\log_e$ transformed. All regression analyses were adjusted for age, sex, impaired glucose metabolism and T2DM, waist, smoking, systolic blood pressure, blood lipids, eGFR, prevalent CVD and medication use.

Results

T2DM was associated with higher GLO1 mRNA and plasma D-lactate levels (β : 0.485 SD; 95% CI: 0.169 to 0.800; $P=0.003$) and (β : 0.340 SD; 95% CI: 0.048 to 0.628; $P=0.023$), respectively), but not with urinary D-lactate levels (β : 0.022 SD; 95% CI: -0.282 to 0.327 $P=0.887$). Overall, we found no consistent associations between GLO1 mRNA and plasma as well as urinary D-lactate and cIMT, ABI or prevalent CVD.

Conclusion

We found that markers of the GLO pathway were higher in T2DM, but were not consistently associated with markers of atherosclerosis. Circulating GLO1 mRNA and both plasma and urinary D-lactate are unlikely to be suitable biomarkers to identify individuals at high risk for CVD in diabetes.

Introduction

Advanced glycation endproducts (AGEs) are a large family of irreversibly sugar-modified proteins which contribute to the development of diabetic complications.¹ We have recently shown that AGEs are formed in atherosclerotic plaques as a consequence of increased metabolic activity and are higher in rupture-prone than in stable human plaques.² Plasma levels of AGEs are also associated with incident cardiovascular disease (CVD) in individuals with type 2 diabetes (T2DM).³

Intracellular sugars and products of glycolysis are potent precursors of AGE formation, and the dicarbonyl methylglyoxal (MGO) is the most reactive AGE precursor.⁴ MGO reacts primarily with arginine residues to form the AGE 5-hydro-5-methylimidazolone (MG-H1). We have recently shown that MG-H1 is increased in rupture-prone plaques, and that MGO-derived AGEs may explain the association between inflammation and growth of the necrotic core in advanced atherosclerosis.²

MGO can be detoxified by glyoxalase 1 (GLO1), glyoxalase 2 and reduced glutathione into D-lactate,⁵ preventing accumulation of MGO and MGO-derived AGEs. GLO1 is considered the rate-limiting enzyme in this reaction.⁶ Indeed, experimental research has shown that GLO1 overexpression in diabetic rats reduces the accumulation of AGEs and also reduces endothelial dysfunction and diabetic microvascular complications,⁷⁻⁹ while knockdown of GLO1 caused development of neuropathy,¹⁰ nephropathy,¹¹ as well as persistent inflammatory changes and activation of the endothelium¹² even in non-diabetic mice. The glyoxalase pathway may thus be one of the major defensive mechanisms against glycation *in vivo* and may be a promising target to protect individuals from diabetic complications such as retinopathy, nephropathy and CVD.

Therefore, individuals with higher expression levels of GLO1 may be relatively protected against diabetic complications. However, the glyoxalase system seems susceptible to dysfunction. For example, Bierhaus et al. showed that expression of GLO1 is decreased in nervous tissue of diabetic mice.¹⁰ Furthermore, expression of GLO1 is decreased in human ruptured plaques² and aortic arches of diabetic ApoE^{-/-} mice.¹³ Although GLO1 expression is regulated by insulin¹⁴ and by the oxidative stress response gene Nrf2,¹⁵ the mechanisms regulating GLO1 expression in diabetes and atherosclerosis have not been completely elucidated. In addition, activity of GLO1 is susceptible to posttranslational modification. We showed previously *in vitro* that conditions of inflammation and hypoxia inhibit GLO1 activity.¹⁶ Several studies have also shown that the enzymatic activity of GLO1 may be inhibited by byproducts of high metabolic activity, such as oxidized glutathione¹⁷ and nitric oxide.¹⁸ However, since we did not find any association between genetic variation of GLO1 and CVD,¹⁹ it remains unclear whether and if so, to what extent expression of GLO1 is associated with diabetes and with CVD. Furthermore, D-lactate is the endproduct of the glyoxalase system, and may serve as a marker for MGO detoxification. D-lactate levels have

indeed been shown to be higher in individuals with T2DM,²⁰ but its association with CVD has not yet been studied.

The aim of the current study was to investigate whether circulating GLO1 mRNA levels, as well as plasma and urinary D-lactate levels, differed between individuals with and without T2DM and were associated with markers of atherosclerotic disease, i.e. plasma markers of endothelial activation and low-grade inflammation, ankle brachial index (ABI), carotid intima media thickness (cIMT) and prevalent CVD.

Research design and methods

Study population

The present study included participants from the follow-up examination of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) Study.²¹ Initial recruitment started in 1999. Of the 574 individuals participating in the initial investigation, 495 (85%) were able to return for a follow-up investigation, which was undertaken from 2006 to 2009.²² Individuals missing data on GLO1 mRNA ($n=21$) were excluded from present analyses, resulting in a total of 474 individuals. Written informed consent was obtained from all participants and the study was approved by the local Medical Ethics Committees.

Measurement of circulating GLO1 mRNA levels

To ensure adequate stabilisation of mRNA, whole blood was collected in a PAX gene tube (Qiagen). Samples were stored at -80°C until further use. mRNA was isolated in an automated fashion with the QIAcube, according to manufacturers instructions. mRNA quantity and quality was assessed with nanodrop. Additionally, RNA integrity number (RIN) was assessed in a subset of samples ($n=216$) to ensure the quality of mRNA was adequate for qPCR (RIN was >5 in 99.5%). Next, 500ng cDNA per sample was synthesized using miScript, according to manufacturers instructions. Next, qPCR of GLO1 was performed. HRPT1 and WHKY1 were used as reference genes, and were not correlated with any of the variables included in our statistical analyses (data not shown).

Primer sequences were for GLO1-forward, 5'-GGTTTGAAGAACTGGGAGTCAAA-3' and for GLO1-reverse, 5'-ATCCAGTAGCCATCAGGATCTTG-3'; for YWHA-forward, 5'-CGTTACTTGGCTGAGGTTGC-3' and for YWHA-reverse, 5'-TGCTTGTTGTGACTGATCGAC-3'; and for HRPT1-forward, 5'-AAG-AAT-GTC-TTG-ATT-GTG-GAA-GA-3' and HRPT1-reverse, 5'-ACC-TTG-ACC-ATC-TTT-GGA-TTA-3'.

Measurement of plasma and urinary D-lactate

Plasma and urine levels of D-lactate were measured with ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as described previously.²⁰

Urinary D-lactate levels were adjusted for urinary creatinine, measured with the Jaffé method, to account for urinary osmolality.

Definition of glucose metabolism status

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.^{21,23} 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 normal glucose metabolism (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 impaired glucose metabolism (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.

Plasma markers of endothelial activation and low-grade inflammation

Endothelial activation was assessed by measuring plasma levels of soluble vascular adhesion molecule (sVCAM-1), soluble intercellular adhesion molecule 1 (sICAM-1), van Von Willebrand factor (vWF) and soluble E-selectin (sE-selectin). Low-grade inflammation was assessed by measuring plasma levels of C-reactive protein (CRP), serum amyloid A (SAA), interleukin 6 (IL-6), interleukin (IL-8), sICAM-1, and tumour necrosis factor (TNF). All these markers were measured with a Meso Scale Discovery multiplex system, except for vWF, which was measured with ELISA.

Markers of atherosclerosis: cIMT, ABI and prevalent CVD

The cIMT was measured in right and left common carotid arteries with an ultrasound scanner equipped with a 7.5. MHz linear array probe and connected to a personal computer equipped with an acquisition system and a vessel wall movement detector software system (Artlab picus, ESAOTE, Maastricht, The Netherlands). cIMT data were available in 453 individuals.

To measure the ABI, systolic blood pressures were measured with a standard Doppler device (Mini Dopplex D900, E-Medical, Harmelen, The Netherlands) in the brachial arteries and twice in both the tibialis posterior and dorsalis pedis arteries of the lower extremities.²¹ The ABI was calculated for each leg by dividing the highest ankle pressure by the highest brachial pressure and the lowest ABI of either leg was used in the analyses.²⁴ We excluded individuals with an ABI > 1.4, which is indicative of arterial

calcification and associated with high cardiovascular risk.²⁵ ABI data were available in 401 individuals.

Previous CVD was defined as the occurrence of one or more of the following: previous myocardial infarction; coronary bypass; percutaneous coronary intervention or stroke reported by questionnaires; signs of myocardial infarction (Minnesota codes 1-1 or 1-2) or ischemia (Minnesota codes 1-3, 4-1, 4-2, 4-3, 5-1, 5-2, 5-3 or 7-1) on a 12-lead electrocardiogram; non-traumatic amputation; and/or an ABI of <0.9. CVD data were available in 474 participants.

Covariates

Study covariates included waist circumference, smoking status, systolic blood pressure (SBP), total- and HDL-cholesterol, triglycerides, estimated glomerular filtration rate (eGFR, using the short Modification of Diet in Renal Disease equation²⁶), and use of blood pressure-, lipid- and glucose-lowering medication, and were determined as described earlier.^{21,23}

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. circulating GLO1 mRNA, plasma and urinary D-lactate levels, all inflammatory markers except sICAM-1) were log_e transformed prior to all analyses. Missing values of covariates were imputed by creating 5 datasets, using multiple imputation. $P < 0.05$ was considered statistically significant.

Diabetes

We used linear regression analyses to investigate the associations between prevalent IGM or T2DM, fasting plasma glucose, HbA1c and levels of circulating GLO1 mRNA, plasma and urinary D-lactate. We adjusted all analyses for age and sex (model 1) and additionally for waist, smoking, SBP, blood lipids, eGFR, prevalent CVD and use of medication (model 2). In model 3 we additionally adjusted for use of insulin, metformin, sulfonylurea derivatives, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) because the promoter of the GLO1 gene has an insulin responsive element,¹⁴ and decreased GLO1 expression can be restored with ARBs in experimental diabetes.²⁷ All results were expressed as standardised coefficients, signifying 1 standard deviation (SD) increase of GLO1 markers relative to the NGM stratum, or per 1 SD increase of fasting plasma glucose or HbA1c.

Circulating markers of endothelial activation and low-grade inflammation

To reduce false positive associations due to the large number of markers included, we calculated scores of endothelial activation by averaging Z-scores of vWF, sICAM-1,

sVCAM-1 and sES and low-grade inflammation by averaging Z-scores of $\log_e$ CRP, $\log_e$ SAA, $\log_e$ IL6, $\log_e$ IL8, sICAM-1 and $\log_e$ TNF. We used linear regression to investigate associations between GLO1 mRNA, plasma and urinary D-lactate and these scores. We adjusted all analyses for age, sex, IGM and T2DM (model 1) and additionally for waist, smoking, SBP, blood lipids, eGFR, prevalent CVD and use of medication (model 2).

Cardiovascular disease

We used linear regression to investigate associations between GLO1 mRNA, plasma and urinary D-lactate and cIMT and ABI. We used logistic regression analyses to investigate the associations between circulating GLO1 mRNA, plasma and urinary D-lactate levels and prevalent CVD. We adjusted all analyses for age, sex, IGM and T2DM (model 1) and additionally for waist, smoking, SBP, blood lipids, eGFR, prevalent CVD and use of medication (model 2).

Next, we investigated whether the associations between the GLO markers (i.e. GLO1 mRNA, plasma and urinary D-lactate) and cIMT, ABI and CVD differed for individuals with and without IGM and T2DM (by adding interaction terms: IGM X GLO marker and T2DM X GLO marker). If an interaction term was $P < 0.1$ we stratified the analysis for the presence of diabetes, using model 2, except that we adjusted for presence of IGM, in the non-diabetic stratum only. All results were expressed as standardised coefficients, signifying 1 SD increase of endothelial activation or inflammation score, cIMT or ABI per 1 SD increase of GLO marker, or 1 increase in odds of prevalent CVD per 1 SD increase of GLO marker.

Results

Baseline characteristics

Table 5.1 shows the baseline characteristics of the participants of the CODAM study. Individuals with T2DM were on average somewhat older, more obese, more often hypertensive and more often had prevalent CVD. GLO1 mRNA, plasma and urinary D-lactate levels were higher in individuals with T2DM. Higher circulating GLO1 mRNA levels correlated with higher plasma D-lactate levels ($\rho_{\text{spearman}}: 0.139, P=0.002$) and higher urinary D-lactate levels ($\rho_{\text{spearman}}: 0.139, P=0.003$).

Table 5.1 General characteristics of the CODAM population stratified according to glucose metabolism status.

	Glucose metabolism status			P-trend
	NGM (n=171)	IGM (n=109)	T2DM (n=131)	
Age (years)	64.7 ± 7.3	66.9 ± 6.9	67.5 ± 6.3	<0.001
Sex (% women)	37.7	42.2	37.1	0.936
Body mass index (kg/m ²)	27.4 ± 3.4	28.2 ± 3.8	30.1 ± 5.0	<0.001
Waist circumference (cm)	97.0 ± 10.3	99.2 ± 11.4	105.4 ± 12.8	<0.001
Fasting glucose (mmol/l)	5.1 ± 0.4	5.6 ± 0.6	7.2 ± 1.9	<0.001
HbA1c (%)	5.9 ± 0.4	6.2 ± 0.4	7.0 ± 0.9	<0.001
Glucose-lowering medication (%)	0.0	0.0	70.7	<0.001
Metformin (%)	0.0	0.0	55.1	<0.001
Sulfonyl-urea derivatives (%)	0.0	0.0	26.3	<0.001
Insulin (%)	0.0	0.0	22.2	<0.001
Total cholesterol (mmol/l)	5.6 ± 1.0	5.8 ± 1.3	5.3 ± 1.2	0.015
HDL cholesterol (mmol/l)	1.4 ± 0.4	1.3 ± 0.4	1.2 ± 0.3	<0.001
Triglycerides (mmol/l)	1.2 (1.0-1.8)	1.6 (1.2-2.0)	1.6 (1.2-2.2)	<0.001
Lipid-lowering medication (%)	23.6	38.8	70.1	<0.001
Current smoking (%)	16.2	12.9	15.0	0.541
Systolic BP (mm Hg)	141.7 ± 20.7	149.6 ± 22.2	150.5 ± 21.8	<0.001
Diastolic BP (mm Hg)	83.0 ± 9.6	84.9 ± 10.7	82.5 ± 10.6	0.638
Mean BP (mm Hg)	102.5 ± 11.8	106.5 ± 13.2	105.2 ± 13.1	0.053
Hypertension ^b (%)	72.3	84.5	89.2	<0.001
BP-lowering medication (%)	43.5	56.9	78.4	<0.001
ARB-blockers (%)	13.1	18.1	25.1	0.004
ACE-inhibitors(%)	12.0	13.8	30.5	<0.001
eGFR (ml/min/1.73m ²)	77.6 ± 16.6	75.3 ± 16.0	80.5 ± 20.4	0.126
Albuminuria ^a (%)	1.0	5.2	9.0	<0.001
Prevalent CVD (%)	31.9	40.5	54.5	0.001
ABI	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	0.995
cIMT	0.81 ± 0.10	0.82 ± 0.11	0.82 ± 0.09	0.479
Endothelial activation score	-0.28 ± 0.77	0.08 ± 1.14	0.27 ± 1.04	<0.001
Low-grade inflammation score	-0.23 ± 0.94	0.11 ± 0.94	0.19 ± 1.03	<0.001
Markers of glyoxalase				
Plasma D-lactate (mmol/l)	6.96 (5.25-9.72)	7.95 (5.91-11.32)	9.56 (6.92-13.45)	<0.001
Urinary D-lactate (mmol/mmol creatinine)	5.20 (2.50-10.40)	4.90 (2.48-9.43)	8.20 (3.60-15.70)	<0.001
GLO1 expression (normalised)	0.86 (0.64-1.09)	0.84 (0.66-1.05)	1.04 (0.86-1.29)	<0.001

Data are presented as means ± SD, medians (IQR) or percentages, as appropriate. ACE-inhibitor, angiotensin converting enzyme inhibitor; ARB-blocker, angiotensin receptor-blocker; CVD, cardiovascular disease; CEL, N^ε-(carboxyethyl)lysine; CML, N^ε-(carboxymethyl)lysine; eGFR, estimated glomerular filtration rate; GLO1, glyoxalase 1; 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. Linear trend was tested with ANOVA, or Chi-square, as appropriate

Associations between presence of diabetes and levels of circulating GLO1 mRNA, urinary and plasma D-lactate

Circulating GLO1 mRNA expression was significantly higher in individuals with T2DM after adjustment for sex and age (β : 0.477 SD (95% CI: 0.272 to 0.683)) and after further adjustment for waist, smoking, SBP, total-to-HDL-cholesterol ratio, triglycerides, albuminuria and use of blood pressure-, glucose- and lipid-lowering medication (β : 0.485 SD (95% CI: 0.169 to 0.805)). When we additionally adjusted the analyses for the major classes of glucose-lowering treatments, i.e. insulin-, metformin-, sulfanyl urea derivatives as well ARBs and ACE-inhibitors, our results remained essentially unchanged (Table 5.2, Model 3). GLO1 mRNA was also significantly associated with plasma glucose and HbA1c in Model 1, but this association was no longer significant after full adjustment (Supplementary Table S5.1, Model 2).

Plasma levels of D-lactate were higher in IGM and T2DM after adjustment for sex and age (Table 5.2, Model 1), but this association remained in the final model only significant in T2DM (β : 0.333 SD (95% CI: 0.037 to 0.620)). Urinary D-lactate was higher as well, in T2DM only (Table 5.2, Model 1), but this was no longer significant in the final model (β : 0.006 SD (95% CI: -0.296 to 0.308)). Higher fasting plasma glucose and HbA1c levels were also associated with higher urinary and plasma D-lactate levels after adjustment for sex and age (Supplementary Table S5.1, Model 1). Results were similar when we further adjusted for the co-variables in Model 2, except for the association between HbA1c and plasma D-lactate, which was no longer significant (Supplementary Table S5.1, Model 2).

Table 5.2 Associations between circulating GLO1 mRNA expression and plasma and urinary D-lactate with IGM and T2DM.

	Model	IGM			T2DM		
		β	95% CI	P	β	95% CI	P
Log ^e GLO1 mRNA	1	-0.063	-0.289 to 0.163	0.586	0.477	0.272 to 0.683	<0.001
	2	-0.128	-0.361 to 0.105	0.280	0.485	0.169 to 0.800	0.003
	3	-0.123	-0.357 to 0.111	0.302	0.488	0.170 to 0.805	0.003
Log ^e Plasma D-lactate	1	0.331	0.108 to 0.553	0.004	0.722	0.520 to 0.924	<0.001
	2	0.137	-0.075 to 0.350	0.205	0.340	0.048 to 0.628	0.023
	3	0.133	-0.081 to 0.346	0.222	0.333	0.037 to 0.620	0.027
Log ^e Urinary D-lactate	1	-0.158	-0.389 to 0.073	0.179	0.416	0.208 to 0.623	<0.001
	2	-0.217	-0.442 to 0.008	0.059	0.022	-0.283 to 0.327	0.887
	3	-0.211	-0.433 to 0.011	0.062	0.006	-0.296 to 0.308	0.969

Data were analysed with linear regression. β , standardized regression coefficient shows the 1SD increase in log^e transformed GLO1 mRNA, plasma or urinary D-lactate in individuals with either IGM or T2DM, relative to individuals with NGM. Model 1: adjusted for age and sex. Model 2: model 1 + waist, smoking, SBP, total-to-HDL-cholesterol ratio, triglycerides, prevalent CVD and use of blood pressure-, glucose and lipid-lowering medication. Model 3: Model 2 + use of insulin, metformin, sulfanyl urea derivatives, ACE inhibitors and ARBs

Associations between markers of the glyoxalase pathway and scores of low-grade inflammation and endothelial activation

Higher levels of GLO1 mRNA were associated with a higher endothelial activation score after adjustment for sex, age, IGM and T2DM (β : 0.147 SD (95% CI 0.059 to 0.235)). Conversely, lower urinary D-lactate levels were significantly associated with a higher endothelial activation score (β : -0.094 SD (95% CI -0.185 to -0.002)). These results did not change in the fully adjusted model (Table 5.3, Model 2). Overall, we found no consistent interactions with prevalent IGM and T2DM.

After adjustment for sex, age, IGM and T2DM, we found an association between higher plasma D-lactate levels and a higher inflammation score (β : 0.128 SD (95% CI 0.038 to 0.218)). However, we found no significant associations after full adjustment for confounders (Table 5.3, Model 2). GLO1 mRNA and urinary D-lactate were not associated with low-grade inflammation in any model.

Associations between markers of the glyoxalase pathway and markers of atherosclerosis.

After adjustment for age, sex, IGM and T2DM, circulating GLO1 mRNA was not associated with cIMT (β : 0.001 SD (95% CI -0.091 to 0.094)), ABI (β : -0.015 SD; 95% CI -0.085 to 0.055)) and prevalent CVD (OR: 1.001 (95% CI 0.819 to 1.223)). Plasma D-lactate was also not associated with cIMT (β : 0.018 SD (95% CI -0.077 to 0.114)) and ABI (β : -0.026 SD (95% CI -0.097 to 0.045)), but we did find an association between higher plasma D-lactate and higher odds of having prevalent CVD (OR: 1.330 (95% CI 1.083 to 1.634)). Urinary D-lactate was also not associated with either cIMT (β : -0.036 SD (95% CI -0.135 to 0.064)), ABI (β : -0.056 SD (95% CI -0.129 to 0.017)) and prevalent CVD (OR: 0.968 (95% CI 0.795 to 1.179)). These results did not change when we further adjusted the analyses (Table 5.4, Model 2).

Next, we investigated whether some of the associations between the GLO markers and markers of CVD differed for individuals with and without diabetes. We found a significant interaction for presence of diabetes in the association between GLO1 mRNA and cIMT ($P_{\text{interaction}}=0.014$). When we stratified the analyses we found no association between GLO1 mRNA and cIMT (β : 0.049 SD (95% CI -0.069 to 0.168)) in individuals without T2DM, but we did find an association between higher GLO1 mRNA levels and lower cIMT levels (β : -0.166 SD (95% CI -0.321 to -0.011)) in individuals with T2DM. All other interaction terms were not significant ($P_{\text{interaction}} > 0.1$).

Table 5.3 Associations between circulating GLO1 mRNA expression and plasma and urinary D-lactate with endothelial activation and inflammation scores.

	Model	Endothelial activation			Low-grade inflammation		
		β	95% CI	P	β	95% CI	P
Log ^e GLO1 mRNA	1	0.147	0.059 to 0.235	0.001	0.070	-0.020 to 0.159	0.126
	2	0.142	0.056 to 0.227	0.001	0.065	-0.021 to 0.151	0.140
Log ^e Plasma D-lactate	1	0.080	-0.010 to 0.170	0.082	0.128	0.038 to 0.218	0.006
	2	-0.005	-0.100 to 0.089	0.910	0.030	-0.064 to 0.125	0.531
Log ^e Urinary D-lactate	1	-0.089	-0.178 to 0.000	0.051	-0.021	-0.111 to 0.068	0.641
	2	-0.094	-0.185 to -0.002	0.045	-0.061	-0.153 to 0.031	0.193

Data were analysed with linear regression. β , standardized regression coefficient shows the 1 SD increase in either ED (sICVAM, sVCAM, sES and vWF) or inflammatory score (CRP, SAA, IL-6, sICAM-1, IL-8 and TNF- α) per 1SD increase in log^e transformed GLO1 mRNA, plasma or urinary D-lactate. Model 1: adjusted for age, sex, IGM and T2DM. Model 2: model 1 + waist, smoking, SBP, total-to-HDL-cholesterol ratio, triglycerides, use of bloodpressure-, glucose and lipid-lowering medication and prevalent CVD.

Table 5.4 Associations between circulating GLO1 mRNA expression and plasma and urinary D-lactate with IMT, ABI and prevalent CVD.

	Model	cIMT			ABI			Prevalent CVD		
		β	95% CI	P	β	95% CI	P	OR	95% CI	P
Log ^e GLO1 mRNA	1	0.001	-0.091 to 0.094	0.977	-0.015	-0.085 to 0.055	0.678	1.001	0.819 to 1.223	0.993
	2	-0.025	-0.118 to 0.068	0.603	0.009	-0.058 to 0.075	0.800	1.012	0.811 to 1.263	0.811
Log ^e Plasma D-lactate	1	0.018	-0.077 to 0.114	0.374	-0.026	-0.097 to 0.045	0.469	1.330	1.083 to 1.634	0.007
	2	0.024	-0.081 to 0.128	0.655	0.033	-0.041 to 0.107	0.377	1.273	1.008 to 1.608	0.042
Log ^e Urinary D-lactate	1	-0.036	-0.135 to 0.064	0.480	-0.056	-0.129 to 0.017	0.130	0.968	0.795 to 1.179	0.746
	2	-0.076	-0.182 to 0.030	0.160	-0.010	-0.084 to 0.064	0.781	1.090	0.871 to 1.365	0.450

cIMT and ABI were analysed with linear regression, CVD was analysed with logistic regression. β , standardized regression coefficient shows the 1 SD increase in either IMT or ABI per 1SD increase of log^e transformed GLO1 mRNA, plasma or urinary D-lactate. OR, standardised odds ratio shows the increase in odds of having CVD per 1 SD increase of log^e transformed GLO1 mRNA, plasma or urinary D-lactate. Model 1: adjusted for age, sex, IGM and T2DM. Model 2: model 1 + waist, smoking, SBP, total-to-HDL-cholesterol ratio, triglycerides, use of bloodpressure-, glucose and lipid-lowering medication and prevalent CVD (analyses cIMT and ABI only).

Discussion

The major findings of this study are that circulating GLO1 mRNA and plasma D-lactate levels were higher in T2DM. This difference was not explained by glucose-lowering treatments, including insulin, or by ARBs and ACE inhibitors. Although we found an association between higher circulating GLO1 mRNA levels and higher levels of endothelial activation markers, circulating GLO1 mRNA levels as well as plasma and urinary D-lactate were not consistently associated with low-grade inflammation scores, cIMT, ABI or prevalent CVD.

In the current study we found that circulating GLO1 mRNA levels are increased in T2DM. This finding was in line with a previous report which reported increased GLO1 activity in circulating cells of patients with type 1 diabetes and complications.²⁸ These findings of increased GLO1 in circulating cells were somewhat unexpected, as we found earlier that GLO1 expression was decreased in ruptured plaques,² and in aortic arches of diabetic mice,¹³ and others have found decreased GLO1 expression in nervous tissue in diabetic mice,¹⁰ and in retinal tissue of diabetic REN-2 rats.²⁷ It is likely that GLO1 mRNA levels in circulating cells do not reflect the GLO1 expression in atherosclerotic plaques. It is possible that GLO1 mRNA is downregulated in the tissues, due to a strong inflammatory milieu,² and that as a compensatory response GLO1 mRNA may be increased in circulating cells.

Since we found that GLO1 expression was only weakly correlated with plasma and urinary D-lactate, this may indicate that expression levels of GLO1 have little influence on MGO detoxification. Therefore, changes in GLO1 activity, or reduced glutathione levels (required for the conversion of MGO into D-lactate by GLO1), may be more important determinants of MGO levels. Indeed, several studies have shown that GLO1 is particularly susceptible to post-translational changes by either oxidized glutathione,¹⁷ nitric oxide,¹⁸ or phosphorylation,²⁹ and most of these modifications have indeed been linked to decreased GLO1 activity. A recent report by Tikellis et al. showed that treatment of mice with a GLO1-inactivating compound indeed increased atherosclerotic lesion size in ApoE deficient mice,³⁰ indicating that very low levels of GLO1 activity aggravate atherosclerosis.

Our finding of increased crude plasma and urinary D-lactate is in line with a previous report,³¹ and with a previous study in which we measured D-lactate in a subset of the baseline examination of CODAM.²⁰ However, we show here that the associations between plasma and urinary D-lactate with T2DM differ considerably when adjusted for potential confounders.

Since GLO1 mRNA was not independently associated with glucose and Hb1Ac (Supplementary Table S5.1), the increase in GLO1 mRNA expression observed does not seem to be a direct consequence of an adaptation to high glucose levels in diabetes. In this study mRNA was isolated from the total population of circulating

cells. Therefore, we cannot fully exclude that the increased GLO1 mRNA expression in T2DM is a reflection of a proportional change in white blood cell subpopulations, because it is unknown to which extent GLO1 mRNA expression differs between white blood cell subpopulations. However, this is unlikely, as increases in circulating cells seem to precede the development of T2DM,³² and we did not observe any increase of GLO1 mRNA in IGM. When we additionally adjusted the associations with T2DM for treatments, such as glucose-lowering medications, insulin, or medications intervening in the renin angiotensin aldosterone system in particular, the associations with T2DM did not change. Therefore, other mechanisms may explain the increased GLO1 expression in T2DM, such as dietary changes in individuals diagnosed with T2DM. Several dietary compounds have been linked to activation of the Nrf2 pathway,³³ which in turn is linked to increased GLO1 expression.¹⁵ In addition, the Nrf2 pathway is activated in response to oxidative stress, by which diabetes is hallmarked.³⁴ Another explanation for the increased expression of GLO1 in T2DM might be that the increased GLO1 mRNA is a direct response to increased MGO levels in T2DM.³⁵ Whether this is also the case in this study remains speculative, as we did not measure MGO in this study. The role of this pathway and other oxidative stress response systems in regulating GLO1 expression in humans should be investigated in future studies.

Interestingly, we did find an association between higher GLO1 mRNA and higher endothelial activation scores. This is a particularly interesting finding, as we and others have shown the protective effects of GLO1 overexpression on endothelial dysfunction³⁶ and microvascular complications,^{37,38} while we and others found that a protective effect of GLO1 overexpression on atherosclerosis was lacking in ApoE deficient mice.^{13,38} However, whether this association was a chance finding should be investigated in an independent cohort study, as we found no consistent associations between plasma and urinary D-lactate and endothelial activation. In addition, we found an association between higher plasma D-lactate levels and higher odds of prevalent CVD. Although we found an interaction with prevalent T2DM for associations between GLO1 mRNA and cIMT, such interactions were not observed for the other markers of atherosclerosis. Taken together, we found no consistent associations between GLO1 mRNA or D-lactate with the endothelial activation and low-grade inflammation scores, ABI, cIMT and prevalent CVD.

This study is to our knowledge the first to investigate whether circulating markers of the glyoxalase pathway are associated with diabetes and CVD. However, the current study has several limitations. Firstly, this study was performed in middle-aged Caucasians at risk of diabetes and CVD. The results of this study may therefore not be applicable to other populations. Nonetheless, our study population represents a large group of individuals in whom activity of the glyoxalase pathway might be relevant in their control of CVD risk. Due to the cross-sectional nature of this study, we cannot exclude that GLO1 mRNA expression precedes prevalence of T2DM (reverse causality), but this is unlikely, as IGM was not associated with GLO1 mRNA. Furthermore, we

measured GLO1 mRNA in circulating white blood cells, but we do not know to what extent this reflects GLO1 activity in tissues, and we cannot exclude that the increased GLO1 mRNA expression reflects a proportional change in circulating cell subpopulations. Although we measured plasma and urinary D-lactate levels, with similar results as with GLO1 mRNA, we cannot fully exclude that D-lactate levels may have been influenced by factors other than MGO levels and/or GLO1 activity.⁵ This may have weakened the associations we studied.

In conclusion, we found that circulating markers of the glyoxalase pathway, i.e. circulating GLO1 mRNA as well as plasma and urinary D-lactate, were increased in T2DM, but were overall not consistently associated with markers of atherosclerosis. For now, circulating GLO1 mRNA and D-lactate in plasma or urine are unlikely to be promising biomarkers to identify individuals who are relatively protected against CVD in diabetes, but this should be confirmed in other (prospective) studies. Furthermore, associations between GLO1 mRNA, plasma and urinary D-lactate may be different for microvascular complications, and this should be addressed in a future study.

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Supplemental table

Table S5.1 Associations between circulating GLO1 mRNA expression and plasma and urinary D-lactate with plasma glucose and HbA1c.

	Model	Fasting plasma glucose			HbA1c		
		β	95% CI	<i>P</i>	β	95% CI	<i>P</i>
Log ^e GLO1 mRNA	1	0.165	0.075 to 0.254	<0.001	0.151	0.060 to 0.243	0.001
	2	0.071	-0.047 to 0.189	0.237	0.054	-0.068 to 0.177	0.385
Log ^e Plasma D-lactate	1	0.305	0.218 to 0.392	<0.001	0.257	0.211 to 0.302	<0.001
	2	0.124	0.017 to 0.231	0.023	0.047	-0.065 to 0.159	0.411
Log ^e Urinary D-lactate	1	0.316	0.230 to 0.403	<0.001	0.300	0.211 to 0.389	<0.001
	2	0.222	0.112 to 0.332	<0.001	0.231	0.117 to 0.345	<0.001

Data were analysed with linear regression. β , standardized regression coefficient shows the 1SD increase in GLO1 mRNA, plasma or urinary D-lactate in individuals with either fasting plasma glucose or HbA1c. Model 1: adjusted for age and sex. Model 2: model 1 + waist, smoking, SBP

Chapter 6

Plasma advanced glycation endproducts are associated with incident cardiovascular events in individuals with type 2 diabetes: a case-cohort study with a median follow-up of 10 years (EPIC-NL)

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Abstract

Experimental data suggest a role for advanced glycation endproducts (AGEs) in cardiovascular disease (CVD), particularly in type 2 diabetes (T2DM). However, epidemiological evidence of an association between high plasma AGEs and increased cardiovascular risk remains inconclusive. Therefore, in a case-cohort study comprising 134 cardiovascular cases and a random subcohort of 218 individuals (including 65 cardiovascular cases), all with T2DM and nested in the EPIC-NL study, plasma levels of protein-bound N ϵ -(carboxymethyl)lysine (CML), N ϵ -(carboxyethyl)lysine (CEL) and pentosidine were measured with liquid-chromatography. AGEs were log_e-transformed, combined in a z-score and the association with incident cardiovascular events was analysed with Cox-proportional hazard regression, adapted for case-cohort design (Prentice method). After multivariable adjustment (sex, age, cohort status, diabetes duration, total cholesterol to HDL-cholesterol ratio, smoking, systolic blood pressure, BMI, blood pressure-, cholesterol- and glucose-lowering treatment, prior cardiovascular events and triglycerides) higher plasma AGE z-scores were associated with higher risk of incident cardiovascular events in individuals without prior cardiovascular events (HR: 1.31 (95% CI 1.06 to 1.61)). A similar trend was observed in individuals with prior cardiovascular events (HR: 1.37 (95% CI 0.63 to 2.98)). In conclusion, high plasma AGEs were associated with incident cardiovascular events in individuals with T2DM. These results underline the potential importance of AGEs in development of CVD.

Introduction

Experimental studies link advanced glycation endproducts (AGEs), a heterogeneous family of sugar-modified proteins, to cardiovascular disease (CVD) and other complications of diabetes.¹⁻³ Several AGE-lowering compounds have been shown to reduce atherosclerosis in animal models,^{4,5} demonstrating that AGEs are no innocent bystanders and are of importance for the development of CVD.

The formation of AGEs is a complex and heterogeneous process, yielding numerous different AGE-adducts, derived from several distinct metabolic processes. AGEs are formed as a result of the Maillard reaction, i.e. a slow and passive process of months, in which sugars react with protein residues on long-lived proteins, leading to cross-linking AGEs such as pentosidine. Pentosidine has been associated with vascular stiffening.⁶ In addition, glucose, and also lipids, can undergo auto-oxidation, leading to AGEs such as N^ε-(carboxymethyl)lysine (CML),⁷ a well-known ligand for the receptor for AGEs (RAGE), which plays a pivotal role in inflammatory signalling.⁸ Furthermore, AGEs are formed during high glycolytic activity, leading to intracellular accumulation of the AGE precursor methylglyoxal,⁹ which reacts with arginine and lysine to form AGEs like 5-hydro-5-methylimidazolone (MG-H1) and N^ε-(carboxyethyl)lysine (CEL). Intracellular formation of AGEs especially takes place in cells that do not regulate their glucose uptake, such as endothelial cells, and induces oxidative stress and endothelial dysfunction.^{10,11}

In addition to previous animal work, it has been shown that AGEs accumulate in human atherosclerotic plaques.^{12,13} Furthermore, levels of AGEs are higher in rupture-prone plaques than in stable plaques, and may explain the association between inflammation and growth of a necrotic core in advanced atherosclerosis.¹⁴ These findings indicate that AGEs may indeed play an important role in the development of cardiovascular events in humans. However, epidemiological studies using plasma measurements of AGEs, which are more feasible than plaque measurements, have generated inconsistent associations with incident cardiovascular events.¹⁵⁻²⁰ These results may be conflicting because they were based on semi quantitative ELISA-based techniques to measure AGEs (measuring mostly one AGE only), instead of specific liquid chromatography based techniques and did not take potential confounding factors (consistently) into account. To address this issue, we investigated associations between plasma AGE levels, measured with liquid chromatography, and prior cardiovascular events and found that AGEs were not associated with prior cardiovascular events, after adjustment for potential confounders, in a large cross-sectional study containing individuals with and without type 2 diabetes (T2DM).²¹ However, in a prospective study among individuals with type 1 diabetes, we did find associations between higher specific plasma AGEs and higher risk of incident cardiovascular events, after adjustment for confounders.²²

Therefore, the aim of the current study was to address whether plasma AGEs, measured with state-of-the-art liquid chromatography, are associated with incident

cardiovascular events in individuals with T2DM. To this end, we measured the major specific protein-bound AGEs CML, CEL and pentosidine in a case-cohort study nested in the prospective EPIC-NL study.

Methods

Study population

The EPIC-NL cohort is the Dutch contribution to the European Prospective Investigation into Cancer and Nutrition (EPIC) and consists of the Prospect-EPIC and MORGEN-EPIC cohorts.²³ In brief, the Prospect-EPIC study includes 17,357 women 49-70 years of age living in Utrecht and vicinity who participated in the nationwide Dutch breast cancer screening program between 1993 and 1997. The MORGEN-EPIC cohort consists of 22,654 men and women 21–64 years of age selected from random samples of the Dutch population in three different towns. Participants were recruited in both studies from 1993 to 1997.

The study population consisted of all participants with confirmed type 2 diabetes at baseline ($n=536$). Three sources of ascertainment of diabetes were used: self-report, hospital discharge diagnoses, and urinary strip test (in the Prospect part of the cohort only). Ascertained cases of diabetes were verified against medical and pharmacy records. Details of the ascertainment sources and verification procedures have previously been described.²⁴

All participants provided written informed consent before study inclusion.

The study complies with the Declaration of Helsinki and was approved by the institutional board of the University Medical Center Utrecht (Prospect) and the Medical Ethical Committee of The Netherlands Organization for Applied Scientific Research Nutrition and Food Research (MORGEN).

Outcome assessment

Follow-up data on incident cardiovascular events were obtained from the Dutch Centre for Health Care Information, which holds a standardized computerized register of hospital discharge diagnoses. All diagnoses were coded according to the ICD-9. Follow-up was complete until the first of January 2008. The database was linked to the cohort on the basis of birth date, sex, postal code, and general practitioner with a validated probabilistic method.²⁵ Information on vital status was obtained through linkage with the municipal registries. Causes of death were collected from Statistics Netherlands, and coded according to the ICD-10. Primary outcome for the present analysis was incident cardiovascular events (fatal and non-fatal). A cardiovascular event was defined as coronary heart disease (CHD), peripheral arterial disease (PAD), congestive heart failure (CHF), and stroke (CVD, 410–414, 427.5, 428, 415.1, 443.9,

430–438, 440–442, 444, 798.1, 798.2, and 798.9; CHD, 410–414, 427.5, 798.1, 798.2, and 798.9; PAD, 440–444; heart failure, 428; and stroke, 430–434 and 436). These end points included both fatal and nonfatal cases. If a participant developed multiple cardiovascular events, the first event was taken into account in the analyses of total incident cardiovascular events. Due to limited statistical power, fatal events were analyzed separately only for total cardiovascular events.

Case-cohort sampling

Case-cohort sampling was performed to reduce costs and preserve valuable biological material. All incident cardiovascular cases ($n=134$) from the baseline patients with type 2 diabetes ($n=536$) were included in this analysis. A random sample of 218 participants was selected to serve as a subcohort in the case-cohort design. Due to the random selection, 65 participants within the subcohort developed an incident cardiovascular event, and overlapped between the subcohort and the cases, an inherent feature of case-cohort studies. 153 participants served as non-cases inside the subcohort, and 69 cardiovascular cases were outside the subcohort.

AGE measurements

Protein-bound plasma AGEs CML and CEL, and lysine, were measured with ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), as described previously.²¹ Protein-bound plasma pentosidine was measured using high performance liquid chromatography (HPLC), as described previously.²⁶ In the present analyses, for CML, CEL, pentosidine and lysine intra-run and inter-run variations were 7.7%, 5.0%, 6.2%, 6.7% and 3.3%, 4.7%, 6.2% and 5.2% respectively. All plasma AGEs were adjusted for lysine, as a marker for total plasma protein.

Other measurements

The general questionnaire addressed demographic characteristics, presence of and risk factors for chronic diseases. Smoking was categorized into current, past, and never smoker. During the baseline physical examination screening, systolic and diastolic blood pressure measurements were performed twice (from which the mean was taken) in the supine position on the right arm using a Boso Oscillomat (Bosch & Son, Jungingen, Germany) (Prospect-EPIC) or on the left arm and using a random zero sphygmomanometer (MORGEN-EPIC). Height and weight were measured by trained staff, and BMI was calculated as weight in kilograms divided by squared height in meters (kg/m^2). Time since diabetes diagnosis was calculated by subtracting the age of diagnosis from the age at baseline examination. Glycated hemoglobin (HbA1c), plasma cholesterol, creatinine and triglycerides were measured,²³ and the estimated glomerular filtration rate (eGFR) was calculated,²⁷ as described previously. All measurements were performed according to standard operating procedures.

Statistical analyses

All analyses were performed using R 2.15.1 for Windows. Missing values on co-variables were replaced using single imputation to reduce bias and increase statistical power.

We $\log_e$ -normalised all plasma AGEs to reduce the potential influence of outliers and to obtain a normal distribution, needed for calculation of z-scores. The AGE score was calculated by averaging (and subsequently standardizing) the z-scores of CEL, CML and pentosidine. To increase statistical power while reducing the risk of chance findings, we performed all primary analyses with AGE score as the exposure.

We investigated the baseline characteristics of the subcohort across tertiles of the AGE-score. Categorical variables were expressed as frequencies. Means and standard deviations (SD) were presented for normally distributed variables, whereas non-normally distributed variables were reported as medians and interquartile ranges (IQR). Linear trend was tested with ANOVA or chi-square, as appropriate. Next, in a combined sample ($n=287$) of the subcohort and cases outside the subcohort, we used a linear regression analyses to investigate the cross-sectional associations of age, sex, $\log_e$ diabetes duration, systolic blood pressure, smoking, total-to-HDL ratio, BMI, blood pressure-, glucose- and cholesterol-lowering treatment, prior cardiovascular events, $\log_e$ triglycerides, glycated hemoglobin (HbA1C), and eGFR with plasma AGEs. These co-variables were adjusted for each other and for cohort, and presented as standardized betas (β s) with 95% confidence intervals (95% CI).

Cox proportional hazards regression models were used to investigate the associations between plasma AGEs with incident cardiovascular events, accounting for the case-cohort design using the method of Prentice.²⁸ In brief, all subcohort members (cases and non-cases) are weighted equally and cases outside the subcohort are not weighted before failure. At time of event, these cases have the same weight as the subcohort members, and only then contribute to the risk sets. Results from a case-cohort study closely resemble analyses on a full cohort.²⁹ All models were adjusted for sex, age, and cohort (Model 1). Furthermore, we additionally adjusted for traditional cardiovascular risk factors, i.e. duration of diabetes, smoking, systolic blood pressure, total-to-HDL cholesterol ratio, prior cardiovascular events, triglycerides, BMI, and cholesterol-, glucose- and blood pressure-lowering treatment (Model 2). As decreased kidney function and poor glycemic control may be potential sources of higher plasma AGEs, we wanted to investigate factors in the causal chain, which we analyzed by adjusting for those variables in a separate step (Model 3). Cox proportional hazards estimates are presented as hazard ratios (HR) and 95% CI. We stratified our main analyses by prior cardiovascular events. By adding interaction terms to model 3, we investigated whether associations were modified by prior cardiovascular events (prior cardiovascular events x AGE) or cohort status (Cohort x AGE). $P < 0.10$ was considered statistically significant for the tests of interaction. Due to limited statistical power, analyses on fatal cardiovascular events, and on CHD, stroke, PAD and CHF were analyzed in the entire population.

Results

Median follow-up time was 10.7 (6.0-12.6) years, 21.6% of the incident cardiovascular events were fatal. Some individuals ($n=19$) developed more than one event, resulting in 150 documented events (80 CHD, 19 stroke, 30 PAD and 21 CHF). Table 6.1 shows the baseline characteristics across tertiles of the AGE score. Higher age, and lower BMI, eGFR and no lipid lowering treatment were significantly associated with higher plasma AGEs in unadjusted analyses. Supplementary Table S6.1 shows the baseline characteristics for the sub-cohort and the cardiovascular cases.

Table 6.1 Baseline characteristics across tertiles of the plasma AGE score in the subcohort ($n=218$).

	Lowest tertile	Middle tertile	Highest tertile	P_{trend}
Participants (n)	73	72	73	-
Female (%)	76.7	80.6	87.7	0.09
Age (years)	56.7 $\pm$ 7.2	58.1 $\pm$ 6.2	59.1 $\pm$ 6.4	0.03
Diabetes duration (years)	3.9 (1.5 - 9.9)	5.3 (2.3 - 10.0)	6.1 (2.9-12.9)	1.00
HbA1c (%)	7.9 $\pm$ 1.6	8.2 $\pm$ 1.7	8.1 $\pm$ 1.6	0.44
HbA1c (mmol/mol)	63.0 $\pm$ 17.5	66.0 $\pm$ 18.6	65.0 $\pm$ 17.5	0.44
Glucose-lowering treatment (%)	91.8	90.3	84.9	0.19
SBP (mm Hg)	141.4 $\pm$ 20.1	141.0 $\pm$ 20.3	142.7 $\pm$ 22.2	0.71
DBP (mm Hg)	83.4 $\pm$ 11.5	81.5 $\pm$ 9.6	81.4 $\pm$ 9.3	0.24
Use of blood pressure-lowering drugs (%)	37.0	34.7	43.8	0.40
Prior cardiovascular events (%)	11.0	19.4	11.0	1.00
Current smokers (%)	30.1	13.9	23.3	0.33
BMI (kg/m ²)	31.5 $\pm$ 4.8	29.7 $\pm$ 4.7	27.1 $\pm$ 4.0	<0.01
Waist (cm)	102.3 $\pm$ 11.6	97.4 $\pm$ 12.1	92.1 $\pm$ 11.2	<0.01
Serum creatinine (umol/l)	58.3 $\pm$ 12.6	58.7 $\pm$ 15.4	67.3 $\pm$ 24.8	<0.01
eGFR (ml/min/1.73m ²)	99.7 $\pm$ 13.1	97.0 $\pm$ 14.9	89.1 $\pm$ 18.0	<0.01
Total cholesterol (mmol/l)	5.3 $\pm$ 1.3	5.2 $\pm$ 1.0	5.1 $\pm$ 1.2	0.26
HDL cholesterol (mmol/l)	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3	1.1 $\pm$ 0.3	0.12
Triglycerides (mmol/l)	2.3 (1.6 - 3.4)	1.9 (1.5 - 3.0)	1.9 (1.0 - 2.6)	1.00
Use of lipid-lowering drugs (%)	8.2	2.8	1.4	0.04
<i>Protein-bound plasma AGEs</i>				
CML (nmol/mmol lysine)	52.1 (46.1 - 59.6)	63.7 (57.8 - 73.1)	80.4 (72.1 - 91.7)	<0.01
CEL (nmol/mmol lysine)	23.5 (20.7 - 26.7)	29.2 (26.0 - 31.9)	32.1 (27.3 - 38.4)	<0.01
Pentosidine (nmol/mmol lysine)	0.5 (0.4 - 0.6)	0.6 (0.5 - 0.8)	1.0 (0.8 - 1.3)	<0.01

Data are presented as frequencies (%), means $\pm$ SD, or medians (interquartile range). HbA1c: glycated hemoglobin, SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, eGFR, estimated glomerular filtration rate, AGEs: advanced glycation endproducts. CML: N^ε-(carboxymethyl)lysine, CEL: N^ε-(carboxyethyl)lysine. Linear trend was tested with ANOVA or chi-square, as appropriate. Skewed variables (diabetes duration, triglycerides and the AGEs) were log_e-transformed prior to analyses.

Determinants of plasma AGEs

Next, we investigated which covariates were independently associated with plasma AGEs. Lower eGFR (β : -0.37 (95% CI -0.49 to -0.24)) was associated with a higher AGE-score, but Hba1c was not associated with plasma AGEs (β : 0.02 (95% CI -0.09 to 0.13)). Glucose-lowering treatment was associated with a lower AGE score (β : -0.39

(95% CI -0.70 to -0.08)). This was also the case for cholesterol- and blood pressure-lowering treatment, but these associations were not statistically significant (Table 6.2). We also found that a lower BMI was associated with a higher AGE-score (β : -0.23 (95% CI -0.34 to -0.12)). Age was also inversely associated with the AGE score (β : -0.14 (95% CI -0.28 to 0.00)) (Table 6.2). We found no association between prior cardiovascular events and the AGE-score. When we analyzed the plasma AGEs separately, we overall found similar results, except for CEL, which was not associated with BMI and glucose-lowering treatment (Table 6.2). These results were similar when we excluded the cases outside the subcohort from analyses (data not shown).

Plasma AGEs and incident CVD

After adjustment for sex, age and cohort, we found a non-significant association between plasma AGEs and incident cardiovascular events, in individuals without (HR: 1.16 (95% CI 0.93 to 1.44)) and with prior cardiovascular events (HR: 1.13 (95% CI 0.73 to 1.74)). After additional adjustment for diabetes duration, total cholesterol to HDL-cholesterol ratio, smoking and systolic blood pressure, prior cardiovascular events, triglycerides, BMI, glucose-, lipid- and blood pressure-lowering treatment (Table 6.3, model 2), a higher plasma AGE score was statistically significantly associated with higher risk of incident cardiovascular events in individuals without prior cardiovascular events, (HR: 1.31 (95% CI 1.06 to 1.61)). A similar trend was observed individuals with prior cardiovascular events (HR: 1.37 (95% CI 0.63 to 2.98)). Additional adjustment for eGFR and Hba1c did not affect these results in individuals without prior cardiovascular events (HR: 1.40 (95% CI 1.10 to 1.78)). Although in individuals with prior cardiovascular events additional adjustment for Hba1c and eGFR seemed to attenuate the association (HR: 0.64 (95% CI 0.30 to 1.35)), there was no statistical interaction between individuals with and without prior cardiovascular events (Table 6.3, model 3).

When we analyzed the AGEs separately, we found overall similar associations for CML (without prior cardiovascular events, HR: 1.47 (95% CI 1.10 to 1.97); with prior cardiovascular events, (HR: 0.98 (95% CI 0.53 to 1.82)) and CEL (without prior cardiovascular events (HR: 1.39 (95% CI 1.12 to 1.72); with prior cardiovascular events, HR: 0.74 (95% CI 0.44 to 1.23)), while pentosidine was not associated with incident CVD events (without prior cardiovascular events, (HR: 1.15 (95% CI 0.89 to 1.49); with prior cardiovascular events, (HR: 0.95 (95% CI 0.59 to 1.54)) (Table 6.3, model 3). The associations between AGEs and incident cardiovascular events in patients without prior cardiovascular events were largely similar to those for the entire population and, although point estimates differed considerably between individuals with- and without prior cardiovascular events in stratified analyses, particularly in Model 3, we did not find any statistical interactions between individuals with- and without prior cardiovascular events (Table 6.3).

Table 6.2 Multivariable Betas and 95% confidence intervals for the cross-sectional associations between covariates and plasma AGEs.

Independent variable	AGE score		CML		CEL		Pentosidine		95% CI		95% CI	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Age (years)	-0.14	-0.28 to 0.00	-0.12	-0.26 to 0.02	-0.13	-0.28 to 0.02	-0.13	-0.28 to 0.02	-0.06	-0.20 to 0.09	-0.06	-0.20 to 0.09
Sex (male is reference)	-0.13	-0.49 to 0.24	-0.28	-0.63 to 0.08	0.16	-0.23 to 0.56	0.16	-0.23 to 0.56	-0.17	-0.53 to 0.19	-0.17	-0.53 to 0.19
Diabetes duration (years)	0.07	-0.04 to 0.18	0.05	-0.06 to 0.15	0.15	-0.03 to 0.27	0.15	-0.03 to 0.27	-0.04	-0.15 to 0.07	-0.04	-0.15 to 0.07
Systolic blood pressure	0.07	-0.04 to 0.19	0.11	0.00 to 0.22	0.04	-0.08 to 0.17	0.04	-0.08 to 0.17	0.01	-0.10 to 0.13	0.01	-0.10 to 0.13
Smoking (non-smoking is reference)	-0.07	-0.21 to 0.07	-0.20	-0.33 to 0.06	0.11	-0.04 to 0.26	0.11	-0.04 to 0.26	-0.06	-0.20 to 0.08	-0.06	-0.20 to 0.08
Total to HDL cholesterol ratio	0.07	-0.06 to 0.19	0.06	-0.06 to 0.18	0.00	-0.13 to 0.14	0.00	-0.13 to 0.14	0.09	-0.04 to 0.21	0.09	-0.04 to 0.21
BMI	-0.23	-0.34 to -0.12	-0.28	-0.39 to -0.17	0.03	-0.10 to 0.15	0.03	-0.10 to 0.15	-0.26	-0.37 to 0.14	-0.26	-0.37 to 0.14
BP-lowering treatment (no is reference)	-0.15	-0.38 to 0.09	-0.20	-0.42 to 0.03	-0.10	-0.35 to 0.15	-0.10	-0.35 to 0.15	-0.04	-0.27 to 0.20	-0.04	-0.27 to 0.20
Cholesterol-lowering treatment (no is reference)	-0.44	-0.98 to 0.10	-0.23	-0.74 to 0.29	-0.75	-1.32 to -0.17	-0.75	-1.32 to -0.17	-0.01	-0.54 to 0.53	-0.01	-0.54 to 0.53
Prior cardiovascular events (no is reference)	-0.18	-0.47 to 0.12	-0.18	-0.46 to 0.10	-0.16	-0.48 to 0.16	-0.16	-0.48 to 0.16	-0.06	-0.35 to 0.24	-0.06	-0.35 to 0.24
Triglycerides (mmol/l)	-0.08	-0.21 to 0.05	-0.20	-0.33 to -0.08	0.13	-0.01 to 0.26	0.13	-0.01 to 0.26	-0.13	-0.23 to -0.03	-0.13	-0.23 to -0.03
Glucose-lowering treatment (no is reference)	-0.39	-0.70 to -0.08	-0.31	-0.61 to -0.01	0.00	-0.33 to 0.33	0.00	-0.33 to 0.33	-0.57	-0.88 to -0.26	-0.57	-0.88 to -0.26
Hba1c (%)	0.02	-0.09 to 0.13	0.04	-0.06 to 0.15	0.00	-0.12 to 0.12	0.00	-0.12 to 0.12	0.00	-0.11 to 0.12	0.00	-0.11 to 0.12
eGFR (ml/min/1.73m ²)	-0.37	-0.49 to -0.24	-0.34	-0.46 to -0.22	-0.15	-0.28 to -0.01	-0.15	-0.28 to -0.01	-0.34	-0.46 to 0.22	-0.34	-0.46 to 0.22

Data were analyzed using linear regression analyses. Diabetes duration and triglycerides were log_e-transformed prior to analyses. β s are expressed per 1 standard deviation increase of independent variable, as 1 standard deviation change of AGE score or log_e-transformed AGE. All independent variables were adjusted for each other and for cohort status.

Therefore, we investigated in the entire population which confounder changed the association between the plasma AGE score and cardiovascular events in Model 1 compared to Model 2 and 3 (Table 6.3). When we omitted BMI from model 3, we no longer found an association between the plasma AGE score and incident cardiovascular events (HR: 1.13 (95% CI 0.89 to 1.44)). We found similar results for CML and pentosidine when we omitted BMI from the model, while CEL remained significant (HR: 1.31 (95% CI 1.04 to 1.67)). In line, when we adjusted for waist circumference instead of BMI, we found near identical results (HR: 1.29 (95% CI 1.01 to 1.66)).

Table 6.3 Associations between plasma AGEs and incident cardiovascular events in the entire population and stratified according to prior cardiovascular events.

	M	Incident cardiovascular events			<i>P</i> _{interaction}
		Entire population (n=287, 134 cases)	No prior events (n=237, 95 cases)	Prior events (n=50, 39 cases)	
AGE score	1	1.11 (0.91 to 1.35)	1.16 (0.93 to 1.44)	1.13 (0.73 to 1.74)	-
	2	1.30 (1.02 to 1.65)	1.31 (1.06 to 1.61)	1.37 (0.63 to 2.98)	-
	3	1.33 (1.03 to 1.71)	1.40 (1.10 to 1.78)	0.64 (0.30 to 1.35)	0.865
CML	1	1.00 (0.81 to 1.24)	1.06 (0.71 to 1.56)	1.07 (0.75 to 1.52)	-
	2	1.31 (0.99 to 1.73)	1.35 (1.05 to 1.73)	1.14 (0.35 to 3.70)	-
	3	1.34 (0.99 to 1.82)	1.47 (1.10 to 1.97)	0.98 (0.53 to 1.82)	0.950
CEL	1	1.21 (0.99 to 1.49)	1.26 (1.00 to 1.58)	1.07 (0.68 to 1.69)	-
	2	1.31 (1.04 to 1.65)	1.31 (1.07 to 1.60)	1.09 (0.59 to 2.00)	-
	3	1.34 (1.06 to 1.70)	1.39 (1.12 to 1.72)	0.74 (0.44 to 1.23)	0.933
Pentosidine	1	1.04 (0.84 to 1.28)	1.07 (0.67 to 1.72)	1.06 (0.75 to 1.51)	-
	2	1.03 (0.80 to 1.33)	1.12 (0.91 to 1.39)	1.24 (0.70 to 2.17)	-
	3	1.02 (0.78 to 1.33)	1.15 (0.89 to 1.49)	0.95 (0.59 to 1.54)	0.450

Data were analyzed using Cox regression analyses. Hazard ratio (HR) is expressed per standard deviation increase of log_e-transformed AGE. Model (M) 1: adjusted for age, sex and cohort. Model 2: model 1 + adjustment for diabetes duration, total cholesterol to HDL-cholesterol ratio, smoking and systolic blood pressure, prior cardiovascular events, triglycerides, BMI, glucose-, lipid- and blood pressure-lowering treatment. Model 3: model 2 + eGFR and HbA1c

Associations between plasma AGEs and specific cardiovascular outcomes

Furthermore, we analyzed the associations between plasma AGEs and the major cardiovascular outcome CHD as well as the less frequent outcomes (fatal cardiovascular events, stroke, PAD and CHF (Supplementary Table S6.2, model 3). Higher plasma AGEs were associated with a significantly higher risk of stroke (HR: 2.06 (95% CI 1.13 to 3.75)) and a borderline significantly higher risk of CHD (HR: 1.32 (95% CI 0.96 to 1.80)). Higher plasma AGEs were also associated with higher risk of fatal cardiovascular events (HR: 1.16 (95% CI 0.85 to 1.62)) and CHF (HR: 1.73 (95% CI 0.77 to 3.91)), but these associations were not significant. In contrast, although non-significant, higher plasma AGEs were associated with lower risk of PAD (HR: 0.76

(95% CI 0.43 to 1.34)). This association was strongest for pentosidine (HR: 0.67 (95% CI 0.40 to 1.13)) (Supplementary Table S6.2, model 3).

Discussion

The main finding of this study was that, after adjustment for confounding factors, higher plasma levels of protein-bound AGEs were associated with higher risk of incident cardiovascular events in individuals with T2DM. Overall, the associations were independent of HbA1c and eGFR. When we analyzed the AGEs separately, we found similar associations with incident cardiovascular events for CML and CEL, but not pentosidine. Results were largely consistent for the subtypes of cardiovascular events, except for PAD, which was inversely associated with the plasma levels of AGEs, and pentosidine in particular.

In line with our previous report,²¹ we again observed no independent association between plasma AGEs and prior cardiovascular events in cross-sectional analyses. In contrast, and in line with our previous work in type 1 diabetes,²² we found that higher plasma AGE levels were associated with increased risk of incident cardiovascular events, particularly after adjustment for confounders (mainly BMI). The lack of associations with prior cardiovascular events may be due to limitations of cross-sectional analyses, such as inclusion of cases only with favourable outcome at baseline. In addition, AGE levels may be susceptible to interventions to treat CVD and may not remain elevated after a cardiovascular event has taken place. This is conceivable, because several lipid-lowering,³⁰ glucose-lowering,³¹ and anti-hypertensive compounds³² have been shown to have AGE-lowering properties and we indeed found that less glucose-lowering treatment was associated with higher plasma AGEs, with similar trends for blood pressure-lowering and lipid-lowering treatment (Table 6.2).

Moreover, obesity seems to have a large influence on AGE-levels. We and others showed an inverse association between BMI and AGEs (CML in particular)^{15,33} and we have postulated trapping of AGEs in tissues in obesity.⁸ This is further substantiated by the current study, as we indeed show that in individuals with T2DM, BMI was inversely associated with plasma AGEs. In fact, only when we adjusted the association of the AGE score with incident cardiovascular events for BMI, we found significant associations. In line, adjusting for waist, another marker for adiposity, yielded identical results. Furthermore, we observed that the direction of the association between age and plasma AGE levels inverted after adjustment for confounders (Table 6.1 vs. 6.2). When we added the confounders separately to the model we discovered this was mainly driven by confounding due to eGFR (data not shown). When we stratified the analyses for presence of prior cardiovascular events, we found, overall, largely similar results between individuals with and without prior cardiovascular events. In model 3 however, we could not detect any significant association in

individuals with prior cardiovascular events. It is unlikely that this is due to a genuine difference between individuals with and without prior cardiovascular events. It is much more likely that this was due to limited statistical power in individuals with prior cardiovascular events, as from the 50 individuals with prior cardiovascular events 39 developed a cardiovascular event and none of the interaction terms were statistically significant. Nonetheless, investigation of the associations between plasma AGEs and incident cardiovascular events in a larger cohort comprising individuals with established CVD would be needed to fully address this question.

With this study, we show that a prospective design and adjustment for confounders has a large impact on the associations between plasma AGEs and incident cardiovascular events (Table 6.3, Model 1 vs. Model 2 and 3).²¹ Therefore, inconsistencies in previous literature may be largely explained by inconsistent adjustment for confounding, as associations with incident cardiovascular events differed greatly with or without adjustment for these confounding factors. Other studies focussing on plasma AGEs and incident cardiovascular events found associations between higher plasma CML and higher risk of incident cardiovascular events in older individuals,^{15,16} while no associations were found in individuals with T2DM.¹⁷ In haemodialysis patients, even associations between higher CML levels and lower risk of incident cardiovascular events have been described.¹⁸ Finally, Kilhovd et al. have found that higher serum levels of AGEs and MG-H1 were associated with higher risk of incident cardiovascular events, but only in non-diabetic women.^{19,20} The majority of previous studies have used stepwise regression models,^{17,18} or have selected co-variables based on significance in univariable analyses.^{15,16} These analyses are less appropriate for etiological analyses, as they are not hypothesis-driven. Thus, selection of the co-variables may be based more on statistical power of the study than on underlying biology and subsequently yield inconsistent models. Indeed, most studies adjusted for BMI,^{15,16,19,20} but others did not,^{17,18} and no study adjusted for glucose- blood pressure- and cholesterol-lowering medication.

We consistently found that CEL in particular was strongly associated with incident cardiovascular events. This finding is in line with the concept that methylglyoxal and its derived AGEs, such as CEL, play the most important role in AGE accumulation¹ and development of diabetic complications.^{34,35} However, this finding should be interpreted with some caution, as for CML and pentosidine, we are likely to underestimate associations with outcome due to residual negative confounding from obesity, as BMI (or waist circumference) does not completely capture adiposity. Furthermore, all associations between plasma AGEs and incident cardiovascular events were independent of HbA1c and eGFR. This is in line with the current concept that AGE accumulation is a complex and heterogeneous process, in which factors such as lipid peroxidation and oxidative stress may play a large role,⁸ and is not simply dependent on hyperglycaemia or decreased renal clearance in diabetes.

Interestingly, higher AGEs (particularly crosslinking AGE pentosidine) were associated with lower risk of PAD (which included abdominal aneurysm related disease).

Although we acknowledge that our current study was underpowered to properly address associations with abdominal aneurysms only, this is a very interesting finding. Crosslinking by AGEs may in fact strengthen the vessel wall, reducing aneurysm formation. These results are in line with a previous cross-sectional study focussing on CML and abdominal aneurysms,³⁶ and may perhaps explain the well-known paradox that individuals with diabetes have an increased risk of CVD, but a lower risk of progression of abdominal aneurysms.³⁷ An adequately powered study should investigate associations between plasma AGEs and development of abdominal aneurysms and PAD as separate endpoints to address this issue.

The present study shows that associations between plasma AGEs and incident cardiovascular events differ considerably from individuals with type 1 diabetes.²² In particular, we found a major confounding effect by obesity, but not HbA1c, and we found no strong positive associations between plasma pentosidine and incident cardiovascular events. Besides their role in CVD, AGEs have been linked to development of microvascular complications of diabetes,¹ but interestingly, some plasma AGEs have been linked to higher, while other have been linked to lower risk of microvascular complications.³⁸ As the associations between plasma AGEs and microvascular complications in T2DM are largely unknown, this should be addressed in a future study.

To the best of our knowledge, our study is the first cohort study investigating associations between plasma AGEs and incident cardiovascular events in T2DM using a large number of cardiovascular cases and state-of-the-art liquid chromatography for assessment of several specific protein-bound AGEs in plasma. Nevertheless, our study has some limitations. As the current study population consists of Caucasians who all had T2DM, our findings may not apply to the general population without diabetes. In addition, although we measured three major specific AGEs, we could not measure the full spectrum of AGEs. Also, as most AGEs are produced intracellularly and/or in tissues, it is most likely that not all AGEs reach the circulation, and we may therefore underestimate the actual strength of the association between AGE accumulation and incident cardiovascular events by measuring plasma AGEs. To this end, AGE measurements that better reflect accumulation of tissue AGEs are needed (such as plasma methylglyoxal levels or AGEs in circulating cells). Furthermore, our study was underpowered to investigate associations between plasma AGEs and specific CVD-outcomes, in particular stroke, PAD and CHF. Therefore, although overall largely consistent, these results should be seen as hypothesis generating. This is particularly true for the protective associations with PAD, which deserve further research. Furthermore, more severe and acute manifestations of CVD, like myocardial infarction or stroke, are easier to diagnose than less severe or chronic manifestations of CVD, such as angina pectoris, CHF, PAD or transient ischemic attack. Also, severe cases are more likely to be treated in the hospital, and thus registered in the Hospital Discharge Registries. Therefore, for outcomes with many minor events, we may underestimate the associations, as misclassification is more likely than for outcomes with more

severe events. This may have resulted in weaker associations,³⁹ which may not necessarily reflect a weaker biological association.

In conclusion, higher levels of plasma AGEs are associated with higher risk of incident cardiovascular events in individuals with T2DM. This study further underlines the importance of AGEs in development of CVD. In addition, future studies are needed to investigate whether AGE measurements, in plasma, circulating cells, or urine, could be developed into biomarkers, to eventually improve risk prediction of CVD, particularly in individuals with diabetes.

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Supplemental tables

Table S6.1 Baseline characteristics for sub-cohort and cardiovascular cases.

	Sub-cohort	Cases
Participants (n)	218	134
Female (%)	81.7	79.3
Age (years)	57.9 ± 6.7	58.6 ± 6.9
Diabetes duration (years)	5.3 (2.3 - 10.2)	6.2 (2.6 - 12.6)
HbA1c (%)	8.0 ± 1.6	8.2 ± 1.7
HbA1c (mol/mmol)	64.0 ± 17.5	66.0 ± 18.6
Glucose-lowering treatment (%)	89.0	80.7
SBP (mm Hg)	141.7 ± 20.8	145.6 ± 23.2
DBP (mm Hg)	82.1 ± 10.2	81.6 ± 11.0
Use of blood pressure-lowering drugs (%)	38.5	48.9
Prior cardiovascular events (%)	13.8	29.6
Current smokers (%)	22.5	31.9
BMI (kg/m ²)	29.4 ± 4.9	30.3 ± 4.9
Waist (cm)	97.2 ± 12.3	99.9 ± 12.7
Serum creatinine (umol/l)	61.5 ± 18.7	64.2 ± 24.3
eGFR (ml/min/1.73m ²)	95.3 ± 16.0	93.3 ± 17.8
Total cholesterol (mmol/l)	5.2 ± 1.2	5.4 ± 1.1
HDL cholesterol (mmol/l)	1.0 ± 0.3	1.0 ± 0.3
Triglycerides (mmol/l)	2.0 (1.4 - 3.1)	2.5 (1.6 - 3.2)
Use of lipid-lowering drugs (%)	4.1	5.2
Protein-bound plasma AGEs		
CML (nmol/mmol lysine)	65.8 (54.2 - 76.8)	65.2 (53.8 - 77.4)
CEL (nmol/mmol lysine)	28.5 (23.7 - 32.2)	28.7 (24.6 - 34.6)
Pentosidine (mmol/mmol lysine)	0.7 (0.5 - 0.9)	0.7 (0.5 - 0.9)

Data are presented as frequencies (%), means ± SD, or medians (interquartile range). HbA1c: glycated hemoglobin, SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, eGFR, estimated glomerular filtration rate, AGEs: advanced glycation endproducts. CML: N^ε-(carboxymethyl)lysine, CEL: N^ε-(carboxyethyl)lysine

Table S6.2 Multivariable adjusted hazard ratios and 95% confidence intervals for the associations between plasma AGEs and specific incident cardiovascular events.

	M	Fatal events	Total CHD	Total Stroke	Total PAD	Total CHF
AGE score	1	1.10 (0.85 to 1.42)	1.13 (0.89 to 1.45)	1.47 (0.98 to 2.19)	0.84 (0.57 to 1.26)	1.03 (0.65 to 1.61)
	2	1.22 (0.90 to 1.65)	1.35 (1.01 to 1.80)	1.95 (1.13 to 3.38)	0.77 (0.45 to 1.31)	1.55 (0.78 to 3.10)
	3	1.16 (0.85 to 1.62)	1.32 (0.96 to 1.80)	2.06 (1.13 to 3.75)	0.76 (0.43 to 1.34)	1.73 (0.77 to 3.91)
CML	1	1.02 (0.68 to 1.53)	0.94 (0.73 to 1.21)	1.70 (1.09 to 2.67)	0.79 (0.54 to 1.17)	0.92 (0.57 to 1.46)
	2	1.32 (0.77 to 2.27)	1.17 (0.85 to 1.61)	2.87 (1.44 to 5.75)	0.86 (0.50 to 1.47)	1.50 (0.72 to 3.11)
	3	1.26 (0.71 to 2.25)	1.09 (0.77 to 1.53)	2.73 (1.31 to 5.70)	0.88 (0.49 to 1.56)	1.59 (0.64 to 3.90)
CEL	1	1.75 (1.19 to 2.58)	1.26 (0.98 to 1.63)	1.37 (0.83 to 2.25)	1.05 (0.72 to 1.53)	1.36 (0.68 to 2.14)
	2	2.03 (1.34 to 3.08)	1.35 (1.02 to 1.79)	1.59 (0.89 to 2.83)	1.05 (0.66 to 1.67)	1.77 (0.93 to 3.36)
	3	2.20 (1.39 to 3.46)	1.37 (1.03 to 1.82)	1.81 (0.97 to 3.40)	1.02 (0.63 to 1.64)	2.07 (0.99 to 4.31)
Pentosidine	1	1.18 (0.80 to 1.74)	1.14 (0.88 to 1.48)	1.20 (0.77 to 1.87)	0.81 (0.53 to 1.23)	0.84 (0.51 to 1.38)
	2	1.29 (0.84 to 1.98)	1.20 (0.89 to 1.62)	1.28 (0.74 to 2.22)	0.66 (0.39 to 1.11)	0.88 (0.43 to 1.77)
	3	1.27 (0.81 to 1.99)	1.14 (0.82 to 1.57)	1.18 (0.63 to 2.22)	0.67 (0.40 to 1.13)	0.81 (0.33 to 1.97)

Data were analyzed using Cox regression analyses. Hazard ratio (HR) is expressed per standard deviation increase of log_e-transformed AGE. Model (M) 1: adjusted for age, sex and cohort. Model 2: model 1 + adjustment for diabetes duration, total cholesterol to HDL-cholesterol ratio, smoking and systolic blood pressure, prior cardiovascular events, triglycerides, BMI, glucose-, lipid- and blood pressure-lowering treatment. Model 3: model 2 + eGFR and HbA1c.

Chapter 7

Higher levels of Advanced Glycation Endproducts in human carotid atherosclerotic plaques are associated with a rupture-prone phenotype

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Abstract

Aims

Rupture-prone atherosclerotic plaques are characterized by inflammation and a large necrotic core. Inflammation is linked to high metabolic activity. Advanced glycation endproducts (AGEs) and their major precursor methylglyoxal are formed during high metabolic activity and can have detrimental effects on cellular function and may induce cell death. Therefore, we investigated whether plaque AGEs are increased in human carotid rupture-prone plaques and are associated with plaque inflammation and necrotic core formation.

Methods and results

The protein-bound major methylglyoxal-derived AGE 5-hydro-5-methylimidazolone (MG-H1) and N^ε-(carboxymethyl)lysine (CML) were measured in human carotid endarterectomy specimens ($n=75$) with tandem mass spectrometry. MG-H1 and CML levels were associated with rupture-prone plaques, increased protein levels of the inflammatory mediators IL-8 and MCP-1 and with higher MMP-9 activity. Immunohistochemistry showed that AGEs accumulated predominantly in macrophages surrounding the necrotic core and co-localized with cleaved caspase-3. Intra-plaque comparison revealed that glyoxalase 1 (GLO1), the major methylglyoxal-detoxifying enzyme, mRNA was decreased (-13%, $P<0.05$) in ruptured compared to stable plaque segments. In line, in U937 monocytes, we found reduced GLO1 activity (-38%, $P<0.05$) and increased MGO (346%, $P<0.05$) production after stimulation with the inflammatory mediator TNF. Direct incubation with methylglyoxal increased apoptosis up to two-fold.

Conclusions

This is the first study showing that AGEs are associated with human rupture-prone plaques. Furthermore, this study suggests a cascade linking inflammation, reduced GLO1, methylglyoxal and AGE-accumulation and subsequent apoptosis. Thereby, AGEs may act as mediators of the progression of stable to rupture-prone plaques, opening a window toward novel treatments and biomarkers to treat cardiovascular diseases.

Introduction

Rupture of an atherosclerotic plaque and subsequent thrombosis is the major cause of cardiovascular events such as heart attack and stroke.^{1,2} The major histological components of rupture-prone plaques are inflammation, macrophage infiltration and a large necrotic core.³

Inflammation of the plaque is characterized by high metabolic activity in macrophages, as characterized by high ¹⁸Fluor-deoxyglucose uptake,⁴ hypoxia⁵ and oxidative stress.⁶ Advanced glycation endproducts (AGEs) are a large family of extensively sugar-modified proteins which can be formed in plaques as a consequence of increased metabolic activity.⁷ Indeed, we and others have demonstrated that AGEs, including major AGE N^ε(carboxymethyl)lysine (CML), are present in atherosclerotic lesions.⁸⁻¹¹ Moreover, animal studies have shown that therapies decreasing AGEs attenuate atherosclerosis.¹²⁻¹⁴ Despite the clear link between AGEs and atherosclerosis, their precise mechanism of action remains largely unknown.

It is becoming apparent that especially intracellular sugars and their derivatives can induce AGE formation, with the dicarbonyl methylglyoxal (MGO) as the most reactive AGE precursor.¹⁵ MGO reacts primarily with arginine residues to form the non-fluorescent AGE 5-hydro-5-methylimidazolone (MG-H1). MGO-derived AGEs have been linked to vascular complications,¹⁶ generation of free oxygen radicals,¹⁷ and apoptosis.^{18,19} MGO can be detoxified by glyoxalase 1 (GLO1), GLO2 and reduced glutathione into D-lactate,²⁰ thereby preventing accumulation of MGO and MGO-derived AGEs. Therefore, we hypothesized that reduced GLO1 activity, production of MGO and subsequent AGEs may be a major factor contributing to the association between plaque inflammation and necrotic core formation, which in turn may predispose plaques to rupture.

In rupture-prone plaques, i.e. plaques with an inflammatory atheromatous phenotype, we found higher plaque AGEs, which accumulated in macrophages, and co-localized with cleaved caspase-3 and hypoxia. Moreover, GLO1 was decreased in ruptured plaques. In line, we showed a direct role of MGO on apoptosis *in vitro*. Taken together, our data suggest that decreased GLO1 and increased MGO and AGEs arise in metabolically active plaques, and may contribute to plaque rupture by inducing apoptosis of macrophages, increasing the size of the necrotic core.

Methods

An elaborate description of the methods is provided in the online supplement.

Athero-Express biobank

This study included a random set of 75 plaques from symptomatic ($n=62$) and asymptomatic ($n=13$) patients undergoing carotid endarterectomy (CEA) from the

Athero-Express biobank. The study was approved by the local ethics committee and written informed consent was obtained from all participants. Plaques of the inflammatory and atheromatous phenotype, i.e. high macrophages and lipid scores, and with low smooth muscle cell scores were classified as rupture-prone ($n=20$). Plaques with a fibrous phenotype, low macrophage and lipid scores, and high smooth muscle cell content were characterized as stable ($n=26$). Plaques not falling in either category were classified as intermediate plaques ($n=29$).

Measurement of AGEs using tandem mass spectrometry

Protein-bound CML, and MGO-derived AGEs N^ε-(carboxyethyl)lysine (CEL) and MG-H1 were measured in homogenates of carotid plaques using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MSMS) by a slightly modified single-run method described earlier.²¹ Intra-assay variation for CML, CEL and MG-H1 was 4.8, 5.0 and 18.1%, respectively. All samples of the Athero-Express were measured in a single run, eliminating inter-run variation.

Protein measurements

Plaque cytokines and chemokines (Table 7.2) were measured by a multiplex suspension array system according to the manufacturer's protocol (Bender Med Systems). Matrix metalloprotease (MMP)-2, MMP-8, and MMP-9 activities were measured using the Biotrak activity assays (Amersham Biosciences).

IL-8, MCP-1 and MMP-9 in cell culture supernatant were measured using Meso Scale Discovery (Gaithersburg, USA) multispot human cytokine assays for tissue cultures according to manufacturer's instructions.

Immunohistochemistry

A random set of 40 plaques from the Athero-Express was selected for immunohistological analysis. Consecutive sections were stained with a mouse monoclonal antibody anti-MG-H1 (1:50.000) and anti-CML (1:4000).¹¹ Horseradish peroxidase-antimouse IgG (Immunologic) was used as a secondary antibody.

In addition, stainings were performed with anti-CD68 (1:500, Sigma), GLO1 (1:2000), and cleaved caspase-3 (1:100, Cell signaling) on carotid plaques, obtained from CEA, from the biobank of the Cardiovascular Research Institute of Maastricht (CARIM), Maastricht University.

Staining for hypoxia (mouse anti-pimonidazole 1:50, hypoxyprobe, NPI) and oxidative stress (mouse anti-nitrotyrosine 1:500, Calbiochem, San Diego, USA) were performed on slides of carotid arteries obtained from 13 patients, infused with pimonidazole prior to CEA.⁵

CARIM biobank (Transcript and protein analysis of GLO1 and RAGE)

An intra-plaque comparison approach consisting of CEA atherosclerotic plaque samples that were selected from the CARIM biobank at Maastricht University, the Netherlands, for transcript and protein analysis was used to compare GLO1 and RAGE mRNA and protein between ruptured and stable plaque segments ($n=26$).

GLO1 activity was assayed by spectrophotometry (Synergy, Biotek) by monitoring the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione for ten minutes at 37°C according to the method of McLellan and Thornalley,²² in homogenates of stable plaques ($n=4$) and plaques with an intra-plaque haemorrhage ($n=6$).

In vitro experiments

Human U937 monocytes (ATCC) were cultured in RPMI 1640 with glutamax, (GIBCO), with 1% penicillin and streptomycin, (GIBCO) and 10% fetal calf serum. Cells were cultured for 24 hours in 0% oxygen and 48 hours in 0.2% oxygen. To study hyperglycaemia, cells were cultured for 14 days in high glucose (30 mmol/l), or mannitol, as an osmotic control.

Cells were stimulated in serum-free culture medium with 0.2% bovine serum albumin, and exposed for 24 hours to tumour necrosis factor (TNF, Sigma) (100 U/ml), aminoguanidine (Sigma) (250 µmol/l), *N*-acetylcysteine (NAC, Sigma) (1 mmol/l), H₂O₂ (1 or 10 µmol/l), and/or to MGO (Sigma) (0.1 mmol/l, 0.2 mmol/l and 0.4 mmol/l). Next, U937 monocytes were exposed for 24 hours to human serum albumin (HSA, 33 µg/ml) and CML-modified HSA. U937 monocytes were transfected with siRNA against GLO1 (Ambion) with AMAXA nucleofactor kit C (Lonza) by electroporation, according to manufacturer's protocol. Apoptosis was assessed by flow cytometry (FACS) using annexin-V and 7-AAD following manufacturers instructions (BD Pharmingen). Cell viability was assessed by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Invitrogen) assay. GLO1 mRNA expression was assayed by qPCR. CML and MG-H1 were quantified in cell lysate with Western blot. B-actin was used as a loading control.

Murine macrophages

Bone marrow-derived murine macrophages were obtained by differentiating femoral and tibial bone marrow suspensions as described previously.²³ Bone marrow was isolated from 10 week old C57BL6 mice (JAX® Mice). Experiments were performed according to Dutch laws, approved by the Committee for Animal Welfare of Maastricht University. At day 9 the macrophages were plated in OptiMEM (GIBCO) and incubated with or without mouse TNF (Peprotech, 500 U/ml) and MGO (0.1 mmol, 0.2 mmol/l and 0.4 mmol/l) for 24 hours. Cell death (SubG1 peak) and

proliferation (S and G2M peak) was assessed by FACS using Propidium-Iodine, following manufacturer's instructions (BD cycletest).

MGO and Glyoxal

Secretion of MGO, as well as glyoxal (GO) in culture medium were measured with UPLC-MSMS.²⁴ Inter-assay variations for MGO and GO were 7.3, 14.3%, respectively. Intra-assay variations for MGO and GO were 2.9 and 4.3% respectively.

Data analysis

Patient characteristics were expressed as absolute and relative frequencies, as mean with standard deviation, or as median with interquartile range, when appropriate. Increases in patient characteristics per plaque category were tested for trend, using analysis of variance (ANOVA) or Chi-square, as appropriate. Next, associations between plaque AGE levels and categories of plaque phenotype and inflammatory markers were assessed with analysis of co-variance (ANCOVA) and linear regression respectively, adjusting for sex, age, smoking and presence of diabetes, to rule out potential confounding by these factors. Skewed variables were log-transformed prior to all analyses. Results from *in vitro* experiments were presented as mean with standard error, and statistical significance of the difference between two groups was tested by students T-test for two independent samples. Probability values <0.05 were considered significant.

Results

CML and MG-H1 plaque levels are higher in rupture-prone plaques

We first investigated whether plaque MGO-derived AGE levels are associated with a rupture-prone phenotype in human carotid plaques. The clinical characteristics of the included patients are shown in Table 7.1.

Significantly higher concentrations of CML and MG-H1, but not CEL, were observed in rupture-prone plaques (Table 7.1), even after adjustment for sex, age, smoking and presence of diabetes (Figure 7.1). Additionally, higher MG-H1 concentrations were observed with increasing percentage of plaque lipid content (Suppl. Figure 7.1A), and a similar trend was observed for CML (Suppl. Figure 7.1B). In line, higher MG-H1 levels were observed in atheromatous lesions compared to fibrous lesion types (Suppl. Figure 7.1A). Interestingly, no increase of plaque AGEs were observed in patients with diabetes ($n=39$) as compared to non-diabetic patients ($n=36$) for CML (79.0 (58.6-126.3) vs. 90.6 (65.6-150.1) nmol/mmol lysine, $P=0.23$), CEL (89.2 (82.0-106.9) vs. 93.4 (75.7-109.0) nmol/mmol lysine, $P=0.97$) and MG-H1 (367.0 (307.0-866.7) vs. 406.2 (288.2-660.0) nmol/mmol lysine, $P=0.95$). In line, we also found no correlation

between plaque levels of CML ($\rho_{\text{Spearman}}=-0.118$, $P=0.40$), CEL ($\rho_{\text{Spearman}}=-0.238$, $P=0.09$) and MG-H1 ($\rho_{\text{Spearman}}=-0.129$, $P=0.36$) with plasma glucose levels. These results did not change when we adjusted for insulin-use and/or glucose-lowering medication in a linear regression analyses.

Table 7.1 General characteristics of individuals, stratified according to plaque phenotype, from the Athero-Express study ($n=75$)

Patient characteristics	Plaque phenotype			P_{trend}
	Stable ($n=26$)	Intermediary ($n=29$)	Rupture-prone ($n=20$)	
Age, years	67 $\pm$ 9	68 $\pm$ 9	69 $\pm$ 8	0.44
Sex (Male), %	69.2	72.4	75.0	0.67
Smoking, %	26.9	37.0	15.8	0.49
Systolic blood pressure, mm Hg	160 $\pm$ 24	150 $\pm$ 32	160 $\pm$ 25	0.97
Diastolic blood pressure, mm Hg	83 $\pm$ 15	81 $\pm$ 13	85 $\pm$ 14	0.67
History of hypertension, %	73.1	75.9	55.0	0.22
eGFR, ml/min/1.73m ²	71.6 $\pm$ 20.8	70.4 $\pm$ 25.6	64.1 $\pm$ 16.8	0.25
C-reactive protein, mg/l	2.1 (0.9 - 4.5)	6.1 (3.0 - 11.4)	5.2 (0.6 - 6.4)	0.59
Plasma glucose, mmol/l	6.4 $\pm$ 2.1	6.5 $\pm$ 2.6	7.7 $\pm$ 2.21	0.15
History of diabetes, %	53.8	51.7	50.0	0.80
Insulin use, %	23.1	24.1	15.0	0.54
Glucose-lowering medication, %	42.3%	37.1%	45%	0.89
Body mass index, kg/m ²	26.3 $\pm$ 3.2	28.2 $\pm$ 4.9	27.4 $\pm$ 4.2	0.39
History of hypercholesterolemia, %	73.1	89.7	50.0	0.13
Total cholesterol, mmol/l	4.4 $\pm$ 0.9	5.1 $\pm$ 1.9	4.7 $\pm$ 1.0	0.71
High density lipoprotein, mmol/l	1.2 $\pm$ 0.4	1.2 $\pm$ 0.3	1.2 $\pm$ 0.4	0.88
Low density lipoprotein, mmol/l	2.4 $\pm$ 0.7	3.2 $\pm$ 1.7	2.8 $\pm$ 0.7	0.53
Triglycerides, mmol/l	1.9 (0.9 - 2.6)	1.3 (1.1 - 2.3)	1.4 (1.0 - 2.1)	0.44
Statin use, %	92.3	82.8	80.0	0.23
Transient ischemic attack, %	26.9	34.5	50.0	0.11
Minor stroke, %	15.4	24.1	10.0	0.68
Stroke, %	3.8	6.9	5.0	0.84
Amaurosis fugax, %	26.9	20.7	5.0	0.06
Retinal infarction, %	0	0	5.0	0.16
Atypical symptoms, %	3.8	3.4	5.0	0.86
Plaque AGEs				
CML, nmol/mmol lysine	70.85 (58.78 - 88.92)	92.30 (65.55 - 140.05)	126.35 (62.40 - 211.08)	<0.05
CEL, nmol/mmol lysine	90.50 (79.98 - 103.15)	91.00 (73.20 - 110.0)	98.50 (87.40 - 109.38)	0.15
MG-H1, nmol/mmol lysine	320.35 (272.72 - 431.30)	392.00 (297.95 - 841.50)	524.70 (346.35 - 965.70)	<0.05

Data expressed as mean $\pm$ standard deviation, as percentage or as median and interquartile range, as appropriate. Statistical testing was performed for trend, using 1-way ANOVA, or chi-square test, as appropriate. Skewed variables were log-transformed prior to analyses

Figure 7.1 CML and MG-H1 levels are higher in plaques with an intermediary and rupture prone phenotype. Concentrations of CML (A), CEL (B) and MG-H1 (C) were measured with UPLC-MS/MS in homogenates of atherosclerotic carotid plaques from the Athero-Express study with a stable, intermediary and rupture-prone phenotype. Data are expressed as geometric mean and 95% confidence interval. Statistical testing was performed for trend, using ANCOVA. Skewed variables were log-transformed. Analyses were adjusted for age, sex, smoking and presence of diabetes

CML, CEL and MG-H1 plaque levels are associated with markers of plaque inflammation

Since AGEs accumulate in areas of inflammatory activation in atherosclerotic lesions,²⁵ we investigated the associations between plaque AGE levels and plaque inflammatory markers. CML, CEL and MG-H1 were positively associated with MMP-9 (Table 7.2). Furthermore, CEL and MG-H1 were positively associated with MCP-1 (Table 7.2), and this was borderline significant for CML. In addition, CML and MG-H1 were associated with IL-8 (Table 7.2). Furthermore, CEL was associated with OPG and MMP-2 (Table 7.2).

CML and MG-H1 accumulate in apoptotic and hypoxic macrophages

Staining for CML and MG-H1 was observed predominantly in macrophages (Figure 7.2, CD68 positive cells; black arrows) surrounding the necrotic core (Figure 7.2, asterisk). In addition, these macrophages were also positive for the apoptotic marker cleaved caspase-3 (Figures 7.2 and 7.3), suggesting that MGO-derived AGEs play a role in the association between plaque inflammation and necrotic core formation. Furthermore, in consecutive sections, MG-H1 and CML also co-localized with hypoxia (pimonidazole) (Figure 7.3), which is particularly associated with the presence of macrophages, inflammation and apoptosis. We also observed co-localisation between CML, MG-H1 and the oxidative stress marker nitrotyrosine (Suppl. Figure S7.2), but unlike caspase-3 and pimonidazole, we also encountered areas where only nitrotyrosine, but not CML or MG-H1, accumulated. In addition to macrophages, endothelial cells often stained positive for CML (Figures 7.2 and 7.3, white arrows) and for MG-H1 (Figures 7.2 and 7.3, white arrows).

Table 7.2 Associations between plaque AGEs and plaque interleukins and MMPs.

	CML			CEL			MG-H1		
	β	95% CI for β	P	β	95% CI for β	P	β	95% CI for β	P
IL-8	0.307	0.077 to 0.537	<0.05	0.118	-0.128 to 0.364	0.34	0.295	0.047 to 0.544	<0.05
MCP-1	0.240	-0.27 to 0.506	0.08	0.275	0.001 to 0.548	<0.05	0.314	0.041 to 0.588	<0.05
MMP-9	0.421	0.196 to 0.647	<0.05	0.290	0.052 to 0.528	<0.05	0.409	0.173 to 0.646	<0.05
IL-6	0.142	-0.105 to 0.388	0.26	-0.021	-0.275 to 0.234	0.87	0.158	-0.105 to 0.421	0.24
RANTES	0.088	-0.222 to 0.397	0.57	0.314	-0.011 to 0.638	0.06	0.230	-0.104 to 0.564	0.17
PARC	-0.023	-0.289 to 0.224	0.80	0.178	-0.084 to 0.440	0.18	0.114	-0.152 to 0.380	0.39
TARC	-0.079	-0.341 to 0.182	0.55	0.057	-0.213 to 0.328	0.67	0.025	-0.249 to 0.298	0.18
OPG	-0.045	-0.644 to 0.553	0.88	0.711	0.118 to 1.305	<0.05	0.319	-0.300 to 0.938	0.31
sICAM	-0.029	-0.291 to 0.232	0.82	0.042	-0.227 to 0.311	0.76	-0.032	-0.305 to 0.241	0.82
MMP-2	-0.014	-0.272 to 0.244	0.91	0.254	0.003 to 0.506	<0.05	0.117	-0.148 to 0.383	0.38
MMP-8	0.157	-0.089 to 0.403	0.21	0.223	-0.020 to 0.466	0.07	0.198	-0.055 to 0.451	0.12

The table shows associations between plaque levels of CML, CEL and MG-H1 with IL-8: interleukin-8, MCP-1: monocyte chemoattractant protein-1, MMP-9: matrix metalloproteinase-9, IL-6: interleukin 6, RANTES: regulated on activation, normal t expressed and secreted, PARC: pulmonary and activation-regulated chemokine, TARC: thymus and activation-regulated chemokine, OPG: osteoprotegerin, sICAM: soluble inter-cellular adhesion molecule, MMP-2: matrix metalloproteinase-2, MMP-8: matrix metalloproteinase-8. Data were analyzed using linear regression, all skewed variables were log-transformed prior to analysis and adjusted for age, sex, smoking and presence of diabetes. The plaque AGEs were the dependent variable. β indicates 1 standard deviation increase of log-transformed AGE per 1 standard deviation increase log-transformed inflammatory marker.

Figure 7.2 CML and MG-H1 co-localization with macrophages (CD68), and apoptosis (cleaved caspase-3). HE stainings, and immunohistochemical detection of CD 68, GLO1, CML, MG-H1 and cleaved caspase-3 were performed in CEA specimens. The HE-staining shows an extensive macrophage infiltration surrounding a large intra-plaque hemorrhage (hashtag) in the necrotic core (asterisk). CML and MG-H1 accumulated predominantly in macrophages (CD68), especially in apoptotic macrophages (cleaved caspase-3) surrounding the necrotic core. Black arrows indicate macrophages, white arrows indicate plaque vessels. The square indicates zoomed areas.

Figure 7.3 CML and MG-H1 co-localize with hypoxic regions. Plaques of patients infused with hypoxia marker pimonidazole were stained for pimonidazole, cleaved caspase-3, CML and MG-H1. We observed a striking co-localization between hypoxia (pimonidazole), CML, MG-H1 and apoptosis (cleaved caspase-3). Black arrows indicate macrophages, white arrows indicate plaque vessels. The square indicates zoomed area.

GLO1 mRNA and protein level, but not RAGE mRNA, are lower in ruptured plaque areas

Underlining the importance of GLO1 in maintaining cell-viability, GLO1 staining was abundantly present in the cytoplasm of virtually all cells of the plaque (Figure 7.2), but not in the necrotic core. Moreover, micro-array data demonstrated that GLO1 mRNA was significantly lower in ruptured plaque-segments when compared to stable plaque-segments (ratio ruptured/stable=0.874; $P<0.01$). In addition, GLO1 protein levels were decreased to a similar extent in ruptured compared to stable segments (ratio ruptured/stable=0.871), although this was not statistically significant. The same was true when we measured GLO1 activity in homogenates of stable and ruptured plaques (ratio ruptured/stable=0.613). In contrast to GLO1, we found no differences in RAGE expression between stable and ruptured plaque segments (ratio ruptured/stable=0.994; $P=0.53$)

Hypoxia and TNF decrease GLO1 activity and increase AGE precursors

Since CML and MG-H1 co-localized with macrophage-rich and hypoxic regions, and we found decreased GLO1 expression and activity in ruptured plaques, we investigated in U937 monocytes whether hypoxia and inflammation can decrease GLO1 activity and induce formation of the major AGE precursors (in particular MGO).

We first investigated whether hypoxia contributed to accumulation of AGEs. U937 monocytes cultured in 0.2% O₂ induced a reduction of GLO1 activity (Figure 7.4A) and increased MGO (Figure 7.4B) and GO (Figure 7.4C) levels. This effect was much stronger when the cells were cultured in 0% O₂ (Suppl. Figure 7.3).

Next, activation of cells with the pro-inflammatory cytokine TNF decreased GLO1 activity (Figure 7.4D), accompanied by increased formation of MGO (Figure 7.4E), and the AGEs MG-H1 (Figures 7.4G and 7.4H) and CML (Figures 7.4I and 7.4J), as well as IL-8 (Figure 7.4K), MCP-1 (Figure 7.4L) and MMP-9 (Figure 7.4M). Similarly, GLO1 activity was also reduced (Suppl. Figure S7.4A), and MGO increased (Suppl. Figure 7.4B), when we stimulated murine macrophages with TNF.

The TNF-induced accumulation of AGEs in U937 monocytes was not dependent on oxidative stress, as co-incubation with NAC did not restore GLO1 activity or reduce MGO or CML (Suppl. Figure 7.5). However, incubation of U937 monocytes with H₂O₂ strongly increased CML (Suppl. Figure 7.6), suggesting that high levels of oxidative stress may act as an independent source of AGEs. In line with the Athero-Express, high glucose did not significantly influence GLO1 activity, MGO or AGE levels in U937 monocytes (Suppl. Figure S7.7).

The inflammatory response of TNF on IL-8, MCP-1 or MMP-9 was not dependent on AGEs, because the AGE-inhibitor aminoguanidine lowered MGO and AGEs (data not shown) but did not decrease IL-8, (Figure 7.4K) MCP-1 (Figure 7.4L) or MMP-9 (Figure 7.4M) secretion. In line, direct incubation of monocytes with CML or MGO did not increase the secretion of IL-8 (Figure 7.4K), MCP-1 (Figure 7.4L) or MMP-9 (Figure 7.4M).

MGO induces apoptosis

Since MG-H1 and CML predominantly accumulated in apoptotic macrophages around the necrotic core, and major AGE precursor MGO is associated with inducing apoptosis,^{18,19} we investigated the effect of MGO on U937 cell-viability. MGO induced formation of MG-H1 (Suppl. Figure S7.8A, S7.8B), and to a lesser extent CML, (Suppl. Figures S7.8C, S7.8D) as well as a dose-dependent increase in AAD-7 and Annexin-V double positive cells, indicating apoptotic cells (Figures 7.5A, 7.5B and 7.5C). In line, MGO induced cell-death in murine macrophages (Suppl. Figure S7.4F), but did not decrease proliferation, as assayed with propidium-iodine (S-peak and G2/M peak) (Suppl. Figure S7.4G, S7.4H).

Figure 7.4 Hypoxia and TNF inhibit GLO1 activity and induce MGO in U937 monocytes. U937 cells were cultured under hypoxic conditions (0.2% O₂) or with TNF (100 U/ml) for 24 hours. GLO1 activity (**A**, **D**) was measured. MGO (**B**, **E**) and GO (**C**, **F**) were measured in the culture supernatant with UPLC-MS/MS. MG-H1 (**G**), quantification (**H**) and CML (**I**), quantification (**J**) were measured with Western-blot in the cell-lysate. β-actin was used as a loading control. U937 monocytes were treated with TNF, aminoguanidine (AG), MGO and HSA-CML, and protein levels of IL-8 (**K**), MCP-1 (**L**) and MMP-9 (**M**) were measured in the supernatant. Data are presented as mean and standard error. Differences were tested with student T-test for two independent samples

We next investigated whether decreased GLO1, as found in the ruptured plaque-segments, reduced cell-viability of U937 monocytes. In untreated cells, siRNA against GLO1 lowered GLO1 activity (Figure 7.5D) and mRNA expression (Figure 7.5E), but did not increase endogenous MGO production (Figure 7.5F) and cell viability (Figure 5.5G). However, when challenged with MGO, the transfected cells displayed decreased

MGO-induced cell viability compared to the scrambled-treated cells (Figures 7.5H and 7.5I), demonstrating that GLO1 is important to protect cells from the cytotoxic effects of MGO.

Figure 7.5 GLO1 knockdown reduces viability after incubation with MGO in U937 monocytes. U937 cells were incubated for 24 hours with 0.1, 0.2, 0.3 and 0.4 mM MGO, and apoptosis was assessed by flowcytometric analyses for Annexin-V and 7-AAD (**A**): control, (**B**): 0.4 mmol/l MGO, quantification in (**C**). Statistical testing was performed for trend, using ANOVA. Next, U937 cells were transfected with siRNA against GLO1, and GLO1 activity (**D**) and mRNA (**E**) were measured. MGO (**F**) was measured with UPLC-MS/MS in the culture supernatant. Next, cells were incubated with MGO for 24 hours and viability was measured by an MTT assay (**G**): 0 mmol/l MGO, (**H**): 0.2 mmol/l MGO and (**I**): 0.4 mmol/l MGO). Data are presented as mean and standard error. Differences were tested with student T-test for two independent samples.

Discussion

Our study shows for the first time that the plaque concentrations of the specific AGEs CML and MG-H1 are associated with inflammatory plaque markers and were higher in rupture-prone plaques (i.e. inflammatory atheromatous lesions). CML and MG-H1

predominantly localized in macrophages surrounding the necrotic core, and were associated with apoptosis and hypoxia. In ruptured plaque segments, GLO1 mRNA and protein concentrations were reduced. In U937 monocytes, TNF and hypoxia decreased GLO1 activity and increased the production of the AGE precursor MGO. MGO induced AGE formation and caused apoptosis, which was enhanced by GLO1 knock-down, providing functional evidence for the involvement of GLO1 in MGO-mediated apoptosis. Taken together, these results suggest that the increase of AGEs and consequences thereof for macrophage cell death may contribute to the transition of stable to rupture-prone plaques.

To our knowledge, this is the first study in which specific AGEs were quantified in homogenates of atherosclerotic plaques using UPLC-MSMS. Several studies have shown with immunohistochemistry that AGEs accumulate in atherosclerotic lesions of the aorta,^{8,26,27} coronary⁹ and carotid arteries using antibodies against CML or glycation-modified proteins. In accordance with previous studies,^{25,26,28,29} we identified the macrophage as the major site for AGE accumulation in the plaque.

AGEs can be derived from several pathways in atherosclerotic lesions. Previous work from our group has demonstrated the importance GLO1 as a critical defense line against glycation *in vivo*.³⁰ We hypothesized that inflammation and hypoxia in atherosclerotic plaques contribute to increased concentrations of AGEs by decreased GLO1 expression. Indeed, in U937 monocytes TNF, a strong pro-inflammatory stimulus, as well as hypoxia, decreased GLO1 activity, accompanied by increased MGO production. Importantly, we found similar effects of TNF on GLO1 and MGO in murine macrophages. In line with a previous report,³¹ high glucose did not materially contribute to accumulation of AGEs in U937 monocytes.

One of the hallmarks of rupture-prone plaques is a large necrotic core. In human carotid plaques, CML and MG-H1 were predominantly present in caspase-3 positive macrophages surrounding necrotic regions of the plaque. Furthermore, MGO induced cell-death in monocytes and murine macrophages, a finding which is consistent with previously reported data.^{18,19} We hypothesize that this may lead to a vicious cycle of inflammation, increased formation of MGO (and AGEs), inducing apoptosis of macrophages, growth of necrotic core, up to the point that plaque rupture occurs. This process may be aggravated by hypoxia and oxidative stress. We showed the importance of GLO1 in protecting cells against toxic effects of MGO by showing decreased viability of U937 monocytes after GLO1 knockdown. Therefore, our current work suggests that GLO1 may protect against plaque rupture. To support this notion, we found decreased expression of GLO1 in ruptured versus stable plaque segments. We hypothesize that interventions aimed at restoring the glyoxalase pathway and/or reducing MGO levels in the plaque, will reduce glycation, and subsequently will reduce cell-death and necrotic core size. Future studies are needed to evaluate this concept *in vivo*.

AGEs such as CML and MG-H1 may be more than mere markers of protein damage by MGO. Many studies have shown the importance of the receptor for AGEs (RAGE) in

the progression of atherosclerosis.^{25,32} In plaques, RAGE is present on macrophages²⁵ and endothelial cells.³² Furthermore, it has been demonstrated that extracellular activation of RAGE by AGEs triggers NF- κ B signaling.³³ Therefore, the increased AGEs we observed in rupture-prone plaques may be important ligands for RAGE *in vivo*, contributing to a vicious cycle between plaque inflammation and AGE accumulation. However, the data from the current study suggest that the formation of AGEs seems to be a consequence, and not a cause of the pro-inflammatory status, because AGEs did not mediate TNF-induced secretion of inflammatory markers, nor directly induced inflammatory markers. Since we did not find any up-regulation of RAGE in ruptured plaque segments, the AGE-RAGE interactions may be of lesser importance than intracellular accumulation of AGEs and subsequent cell-death in plaque rupture in human atherosclerosis. These findings are provocative, and deserve further research. In addition, AGEs may contribute to the development of atherosclerosis via the formation of extracellular cross-linking of proteins, as AGE-crosslink breaker ALT-711 attenuates diabetic atherosclerosis in ApoE knock-out mice.¹³ Furthermore, induction of oxidative stress by MGO³⁰ may also contribute to predisposition towards plaque-rupture, as MGO and ROS formation are closely intertwined,³⁴ and it has been showed that MGO induces apoptosis in a ROS-dependent manner.³⁵

Our study has some limitations. First of all, by the cross-sectional design of this study, it is impossible to investigate whether accumulation of AGEs precedes development of rupture-prone lesions. Moreover, in the plaques of the Athero-express study, we defined inflammatory atheromas as rupture-prone plaques, as these plaques have a similar phenotype as ruptured plaques. We cannot determine in this study if these plaques indeed have the highest risk of plaque rupture, although the finding of higher plaque AGEs in rupture-prone plaques is consistent with the decreased GLO1 in the ruptured plaques of the CARIM biobank. Furthermore, we cannot exclude residual confounding in the associations between plaque AGEs and plaque phenotype, as data on potential confounders were either not determined in this study (such as HbA1c), or missing in a high proportion of participants (plasma lipids). We found no increase of plaque AGEs in individuals with diabetes. This finding should be interpreted with caution, as we did not measure HbA1c, and 41% of individuals with diabetes used insulin, and 79.5% used oral glucose lowering medication. However, when we adjusted our analyses for insulin and oral glucose lowering medication our results were similar.

Taken together, our study supplies unique data, showing an association between human plaque concentrations of specific MGO-derived AGEs and GLO1, cell-death, and plaque phenotype. The glycation pathway may be a major player in the progression of stable to rupture-prone plaques and subsequent plaque rupture. Future studies should focus on a causal relationship between AGEs, MGO and plaque phenotype.

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Detailed supplementary Methods

Athero-Express Biobank

Athero-Express is an ongoing longitudinal cohort study, initiated in 2002 by 2 Dutch hospitals: the University Medical Center Utrecht and the St Antonius Hospital in Nieuwegein.¹ The study has been approved by the institutional boards of both hospitals, and written informed consent was obtained from all participants. The study was designed to investigate the expression of atherosclerotic tissue-derived biological markers in relation to plaque phenotype and adverse cardiovascular events during follow-up of patients undergoing carotid endarterectomy (CEA).

Patient Inclusion

In this study a random set of 75 plaques from symptomatic ($n=62$) and asymptomatic ($n=13$) patients from the Athero-express biobank were included. The indication for CEA for asymptomatic patients was based on the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST) and for symptomatic patients was based on recommendations based on the European Carotid Surgery Trial (ECST) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET).²⁻⁵ All patients were reviewed by the vascular surgeon or neurologist before CEA to assess the nature and timing of clinical symptoms. Patients undergoing CEA completed questionnaires covering cardiovascular risk factors, medical history, life style and medication use. The definitions of hypertension, hypercholesterolemia, and diabetes mellitus were restricted to those cases requiring medical treatment.⁶ These data were complete in all participants, except for smoking (missing $n=3$). Further clinical data, such as plasma lipids (total-, HDL-, LDL-cholesterol and triglycerids, missing $n=46$), office measurement of blood pressure (missing $n=5$) and body mass index (missing $n=8$) were obtained from the patient charts.

Plaque Processing

All carotid plaques were carefully dissected from the carotid arteries and immediately transferred to the laboratory for further processing as described previously.¹ In short, the atherosclerotic fragments were dissected by a dedicated technician into 0.5-cm-thick cross-sectional segments along the longitudinal axis of the vessel. The plaque segment showing the largest plaque burden was called the culprit lesion and was used for histological analysis. The definitions of each staining category on which definition of plaque stability was based (H&E, Elastin von Gieson, picrosirius red, [alpha]-actin, and CD68) have been described previously.¹ Subsequently, plaques of the atheromatous phenotype (collagen score 0 and 1; lipid score 2), rich in macrophages (score 2 and 3) and with low smooth muscle cell scores (0 and 1) were classified as rupture-prone plaques ($n=20$). Plaques with the fibrous phenotype (collagen 2 and 3;

lipid score 0 and 1), low macrophage scores (0 and 1), and high smooth muscle cell scores (2 and 3) were characterized as stable ($n=26$). Plaques not falling in either category were classified as intermediate plaques ($n=29$) (Supplementary Table S7.1).

Protein array

We measured a panel of cytokines, chemokines and matrix metalloproteases (MMPs) (Table 7.2). Levels of plaque interleukins were measured by a multiplex suspension array system according to the manufacturer's protocol (Bender Med Systems). Matrix metalloprotease (MMP)-2, MMP-8, and MMP-9 activities were measured using the Biotrak activity assays (Amersham Biosciences).

Measurement of AGEs using tandem mass spectrometry

Levels of AGEs N^ε-(carboxymethyl)lysine (CML), N^ε-(carboxyethyl)lysine (CEL) and 5-hydro-5-methylimidazolone (MG-H1) were measured in homogenates of carotid plaques using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MSMS) by a single-run method described earlier.⁷ In short, 25 μ l of plaque homogenate was incubated for 2 hours with 50 μ l H₂O and 250 μ l Sodium borohydride at room temperature. Next, the solution was precipitated with 1ml of 20% TFA and subsequently centrifuged for 20 minutes at 4°C and 4300 g. The supernatant was removed and the remaining pellet was hydrolysed with 1 ml 6N HCl for 16 hours at 110°C. After hydrolysis 40 μ l hydrolysate and 20 μ l internal standard (containing 1432 nmol/l [²H₂]-CML, 1378 nmol/l [²H₄]-CEL and 1322 nmol/l [²H₃]-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 μ l 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 μ l water. For validation experiments 25 μ l of 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 to 40 μ l of hydrolysate and further prepared as described above. The acid stability was checked for CML, CEL and MG-H1. CML and CEL showed complete recovery after acid hydrolysis. MG-H1 showed a recovery of 60% percent after 16 hours of hydrolysis. The levels of AGEs were corrected for levels of lysine measured with UPLC-MSMS. Inter-assay variation as determined by replicate analysis of plasma samples on 7 different days was for CML, CEL and MG-H1 respectively 7.0, 9.7 and 12.8%. Intra-assay variation as determined by replicate analysis ($n=10$) of a plasma sample on one day was for CML, CEL and MG-H1 respectively 4.8, 5.0 and 18.1%. All samples of the athero-express were measured in a single run, eliminating inter-run variation. In previous validation studies of several sample matrices containing high fat content, we found no differences in the concentrations of AGEs when the samples were treated with- or without methanol-chloroform to extract lipids,⁸ indicating that lipid-peroxidation during tissue processing did not influence levels of CML, CEL or MG-

H1. Therefore, due to the small sample volumes and possible protein loss during liquid liquid extraction, we omitted this sample processing step.

Immunohistochemistry CML and MG-H1

A random set of 40 plaques, from the total 75 plaques included in the present study, was selected for immuno-histological analysis. Consecutive sections were stained with a mouse monoclonal antibody anti-MG-H1 (1:50000) and anti-CML (1:4000)⁹, HRP-antimouse IgG (Immunologic) was used as a secondary antibody. The signal was visualized using diaminobenzidine. Sections were counterstained with hematoxylin.

In addition, stainings were performed with anti-CD68 (1:500, Sigma), GLO1 (1:2000), and cleaved caspase-3 (1:100, Cell signaling) on carotid plaques, obtained from CEA, from the biobank of the Cardiovascular Research Institute of Maastricht (CARIM), Maastricht University.

Next, we performed stainings for hypoxia (mouse anti-pimonidazole (1:50, hypoxyprobe, NPI), oxidative stress marker nitrotyrosine (1:500, Calbiochem, San Diego, USA), MG-H1 and CML in consecutive slides of carotid arteries obtained from 13 patients, infused with pimonidazole prior to CEA.¹⁰ For all immunohistochemical stainings, slides incubated without the first antibody were included as a negative control.

CARIM biobank (Transcript and protein analysis GLO1 and RAGE)

A same-plaque comparison approach consisting of CEA atherosclerotic plaque samples that were selected from the CARIM biobank for transcript and protein analysis was used to assess GLO1 mRNA and protein in ruptured and unruptured plaque segments. Collection, storage and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (<http://www.fmwv.nl>).

We were able to obtain stable-ruptured pairs of samples from 26 patients. The entire surgically removed carotid plaque piece was cut into parallel, transverse segments of 2-3 mm length. Each alternating segment was snap frozen for the cross-omics analysis. The flanking segments were fixed in formalin and processed for histological evaluation. Hematoxylin-Eosine (HE) stained slides from these flanking segments were evaluated for plaque stage using the Virmani classification criteria,¹¹ by a pathologist and an experienced researcher in cardiovascular pathology. Segments designated as stable featured either a fibrous cap atheroma (AHA classification grades IV or V) or pathological intimal thickening (AHA classification type III). While each segment designated as ruptured included a thrombus or presented intra-plaque hemorrhage (AHA classification type VI lesion).¹² Segments were further analyzed when the same patient could provide both a ruptured and a stable piece, according to our above-described criteria.

Tissue samples were stored at -80°C for transcript and protein analyses after pulverizing them first, and then weighing out 5-20 mg doses for each analytical platform depending on the available tissue amount and the needs of the analytical process. As not every primary sample was sufficient for all these analyses, the following platform priority was established: transcript analysis was applied for 52 individual slices ($n=26$) of which 44 slices ($n=20$) met the RNA quality criteria; proteomics measurements could be carried out on 49 slices ($n=23$). Residual amounts of pulverized tissue specimens were used to create a single reference pool that was used for the proteomics and metabolomics measurements. The contributions of stable and ruptured specimens to this reference pool were approximately equal.

Transcriptomics

We isolated the RNA of the 26 sample pairs and determined the RNA quality and integrity using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer. Samples that had an RNA Integrity Number (RIN) lower than 5.6 were excluded. The average RIN was 7.23 ± 0.48 . In total 20 sample pairs were eligible for submission to the transcriptomics platform (Illumina Human Sentrix-8 V2.0 BeadChip®). This platform measures a total of 24,526 human transcripts and variants, as defined by RefSeqs (NCBI) sequences, following manufacturers protocol.

Proteomics

The quantitative discovery proteomics workflow was based on multi-dimensional liquid chromatography combined with multiplex stable isotope labeling technology using the 8-plex iTRAQ reagent.¹⁰ Matched pairs of stable and ruptured plaque samples were arranged into the 8-plex iTRAQ mixes in a way that three plaque pairs and two replicates of the reference sample made up an iTRAQ experiment. This design minimizes error propagation of the relative quantification since no samples across different iTRAQ mixes need to be compared. The study (49 primary samples, 25 reference samples) consisted of the analysis of nine iTRAQ mixes.

iTRAQ mixes were generated by digesting the individual protein samples into peptide pools, labeling them with the iTRAQ reagent and combining them. The mixed peptide pools were fractionated by strong cation exchange chromatography and the resulting fractions were further separated by HPLC. HPLC fractions were spotted onto MALDI plates and analyzed by MALDI MS/MS. Using in-house developed software tools it was ensured that highly consistent sets of peptides and proteins were measured in each of the iTRAQ experiments. After consolidation of the peptide measurements, assignment of peptides to a minimum non-redundant protein set, from the analysis of nine different iTRAQ mixes, a total of 1359 proteins were detected. 1190 of these proteins were detected in a sufficient number of samples to be used for statistical data analysis. Measured values of protein expression were normalized using a procedure based on Van de Sompele et al.¹³

GLO1 activity assay

We homogenized stable ($n=4$, mean age 77.3 ± 6.7 ; 75% men) and ruptured plaques (intra-plaque hemorrhage) ($n=6$, mean age 66.8 ± 8.3 ; 66% men) obtained from CEA to confirm if GLO1 activity was decreased in ruptured plaques. Plaques were deep-frozen, using liquid nitrogen, and crushed with a steel mortar. Subsequently, plaques were dissolved in GLO1 assay buffer. GLO-I activity was assayed by spectrophotometry according to the method of McLellan and Thornalley, monitoring the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione for 30 min at 25°C.¹⁴ GLO1 activity was adjusted for sex and age of the individuals, and for the amount of protein measured in the sample, measured using the Pierce® BCA Protein Assay Kit (23227, Thermo Scientific, Etten-Leur, Netherlands). GLO1 activity was expressed as units per mg protein. 1 unit signifies formation of 1 nmol/l S-D-lactoylglutathione per minute.

In vitro experiments

Human U937 monocytes were cultured in RPMI 1640 with glutamax (GIBCO), with 1% penicillin and streptomycin, (GIBCO) and 10% fetal calf serum, unless stated otherwise.¹⁵

Anoxia and hypoxia

Cells were seeded in a 6 wells plate at 5×10^5 cells/ml in complete culture medium and incubated for 24 hours in 0% oxygen and for 48 hours at 0.2% oxygen. Viability of the cells was ensured with Trypan-blue staining.

High glucose

Cells were cultured in 25 cm² flasks in growth medium with high glucose (30 mmol/l) for 14 days. Mannitol was used as an osmotic control. As a positive control, we performed FACS analyses with the oxidative stress probe 5-(and-6-)-chloromethyl-2'7'-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H₂DCFA), according to manufacturers instructions (C6827, Invitrogen, USA).

TNF stimulation

Cells were seeded in a 12-wells plate with serum free culture medium, with the addition of 0,2% bovine serum albumin, at a density of 5×10^5 cells/ml, and exposed for 24 hours to tumour necrosis factor (TNF, Sigma) (100U/ml), with and without AGE quencher aminoguanidine (Sigma) (250 µmol/l), and with- and without glutathione precursor N-acetyl-cystein (NAC, Sigma) (1 and 5 µmol/l).

H₂O₂ incubation

To induce high levels of oxidative stress, cells were seeded in a 12-wells plate with serum free culture medium, with the addition of 0,2% bovine serum albumin, at a density of 5×10^5 cells/ml, and exposed for 24 hours to 1 or 10 $\mu\text{mol/l}$ H₂O₂.

CML-modified human serum albumin

Cells were stimulated with CML bound to human serum albumin, which was prepared as described previously.⁹ 20 mg pyrogen-free human serum albumin, obtained from the central laboratory of blood transfusion, Amsterdam, was dissolved in 0.5 mol/l phosphate buffer pH 8, and was incubated with glyoxylic acid (3 mmol/l) and 2 mol/l NaCNBH₃ for 24 hours at 37°C. These reactions were terminated by extensive dialyses against milliQ. The degree of CML modification was assessed using UPLC-MSMS and by Western-blot analyses. Cells were seeded in a 12-wells plate with serum-free culture medium, at a density of 5×10^5 cells/ml, and exposed for 24 hours to control albumin (33 $\mu\text{g/ml}$), and CML-modified human serum albumin (33 $\mu\text{g/ml}$).

Incubation with MGO and GLO1 siRNA

Cells were seeded in a 12-wells plate with serum free culture medium, with the addition of 0,2% bovine serum albumin, at a density of 5×10^5 cells/ml, and exposed for 24 hours to MGO (0.1, 0.2, 0.3, 0.4 mmol/l). Next, U937 cells were transfected with siRNA against GLO-1 (Ambion) with AMAXA nucleofector kit C (Lonza), according to manufacturers protocol for U937 cells. After 24 hours cells were incubated with 0.2 and 0.4 mmol/l MGO for 24 hours in serum free medium with 0.2% BSA.

Western blot CML and MG-H1

CML and MG-H1 were quantified in the cell lysate, using Western blot to conserve protein, as protein content required was lower than for UPLC-MSMS. Primary anti-CML was diluted 2000 times, and anti-MG-H1 was diluted 100.000 times. β -actin (1:1000) was used as a loading control.

GLO1 gene expression

GLO1 mRNA gene expression was assayed by Real-Time quantitative PCR (qPCR) performed on a Bio-Rad MyiQ, software IQ5 v2 (Bio-Rad, Hercules, CA, USA) using IQ SYBR Green Supermix with fluorescein (170-5006CUST, Bio-Rad, Hercules, CA, USA) and 10 ng total cDNA. B-Actin was used as reference gene. Normalized gene expression was expressed relative to control. Primer sequences were: B-act-forward, 5'-GTTGTCGACGACGAGCG-3', B-act-reverse, 5'-GCACAGAGCCTCGCCTT-3', GLO1-forward, 5'-GGTTTGAAGAACTGGGAGTCAAA-3', GLO1-reverse, 5'-ATCCAGTAGCCATCAGGATCTTG-3'.

Murine bone marrow derived macrophages

Bone marrow was isolated from 10 week old male C57BL6 mice (002052, JAX[®] Mice, Maine, USA).¹⁶ Animals were sacrificed by carbon dioxide inhalation, and femur and tibia were collected for stem cell isolation. Murine bone marrow derived macrophages were obtained by differentiating femoral and tibial bone marrow suspensions, plated at 30×10^6 cells in 15 cm plates in culture medium: (Gibco[®] RPMI 1640 containing 25 mM HEPES, 15% L929 conditioned medium, 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin). At day 9, the differentiated macrophages were plated at 0.5×10^6 cells/ml in Opti-MEM (GIBCO 31985) and incubated with or without mouse TNF- α (Peprotech, 500 U/ml) and MGO (0.1 mmol/l, 0.2 mmol/l and 0.4 mmol/l) for 24 hours.

Measurement of IL-8, MMP-9 and MCP-1 in culture supernatant

IL-8, MCP-1 and MMP-9 in culture supernatant were measured using Meso Scale Discovery (Gaithersburg, USA) multispot human cytokine assays for tissue cultures, according to manufacturers' instruction.

MGO and Glyoxal

Secretion of MGO, as well as glyoxal (GO), were measured with UPLC-MSMS in the culture medium.¹⁷ Glyoxal (GO) solution (~40%) and methylglyoxal (MGO) solution (~40%) were obtained from Sigma-Aldrich, d_8 -O-phenylenediamine (oPD) (98.6%) was obtained from CDN-isotopes and 3-deoxyglucosone (3-DG) was obtained from Santa Cruz. All other reagents and solvents were of analytical or ULC/MS grade. Internal standards for GO and MGO were not commercially available. A derivatized internal standard was therefore prepared by adding 140 μ l GO (17.5 mmol/l), 40 μ l MGO (6.5 mmol/l), 50 ml water, 500 μ l formic acid and 25 mg d_8 -oPD in a plastic tube. 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. Before analysis, the culture medium was thawed and mixed thoroughly. 50 μ l culture medium was mixed with 100 μ l 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 μ l of internal standard solution was added. Samples were mixed and subsequently centrifuged for 20 minutes at 14000 rpm at a temperature of 4°C. 10 μ l was injected for UPLC/MSMS analysis. Derivatized GO and MGO 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, 50x2.1 mm, 1.7 μ m) with a linear gradient of 5 mmol/l formic acid and acetonitril at 30°C. The flow rate was 800 μ l/min and the injection volume was 10 μ l. Quantification of GO and MGO 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 and MGO were respectively 131.1>77.1 and 145.1>77.1. The MRM transitions for the internal standards [$^2\text{H}_4$]-GO and [$^2\text{H}_4$]-MGO were respectively 135.1>81.1 and 149.1>81.1. 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. Linearity was determined by adding standard solution of GO and MGO to water and plasma matrix. A six-point calibration curve was prepared for GO (1458-0 nmol/l) and MGO (1083-0 nmol/l). The peak area ratio of GO and MGO multiplied by the concentration of each corresponding internal standard were plotted as a function of the concentration. The calibration curves for GO and MGO were linear over the described concentration ranges ($r^2 > 0.99$) in both water and plasma matrix. Mean slope (response factor) for GO and MGO tested in 6 different matrices were respectively 1.036 (CV, 7.5%) and 0.850 (CV, 8.2%). Inter-assay variation as determined by replicate analysis of a plasma sample on 11 different days was for GO and MGO respectively 14.3 and 7.3 %. Intra-assay variation as determined by replicate analysis ($n=10$) of a plasma sample on one day was for GO and MGO respectively 4.3, 2.9%. The lower limits of quantification ($S/N=6$) on column for GO and MGO 200 and 17 fmol, corresponding to a concentration of 100, 9 and 5 nmol/l.

Apoptosis and Cell viability

Apoptosis was assessed in U937 cells by flow cytometry analyses for annexin-V and 7-AAD following manufacturers instructions (BD Pharmingen). We assayed cell-death (SubG1 peak) and proliferation (S and G2/M peak) with Propidium-Iodine (SubG1 peak) in the murine macrophages following manufacturers instructions (BD cycletest), as these are strongly adhering cells, and become false-positive for Annexin-V upon cell-scraping.

In addition, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Invitrogen) assay was performed to assess cell-viability. Briefly, cells were seeded at a density of 5×10^5 /ml cells per well into 96-well culture plates. After 24 hours, cells were incubated with MTT for 4 hours and lysed with hydrogen chloride/isopropanol. Subsequently, absorbance was measured at 570 nm, and at 690 nm for background subtraction.

Data analysis

Patient characteristics were expressed as either absolute and relative frequencies, mean with standard deviation, or as median with interquartile range, when appropriate. Increases in patient characteristics per plaque category were tested for trend, using analysis of variance (ANOVA) or Chi-square, as appropriate. Next, associations between plaque AGE levels and categories of plaque phenotype and inflammatory markers were assessed with analysis of co-variance (ANCOVA) and linear regression respectively, with the plaque AGEs as outcome measurements,

adjusting for sex, age, smoking and presence of diabetes, to rule out potential confounding by these factors. We calculated Spearmans correlations between the plaque AGEs and plasma glucose. To rule out potential confounding by glycemic control, we additionally performed linear regression analyses between plaque AGEs and plasma glucose, with the plaque AGEs as the outcome variable, adjusting for glucose lowering treatment and/or insulin use. Skewed variables were log-transformed prior to all analyses. Results from *in vitro* experiments were presented as mean with standard error, and statistical significance of the difference between two groups was tested by students T-test for two independent samples. Two-sided probability values <0.05 were considered significant. All analyses were performed with SPSS, version 20, except for the *in vitro* experiments, which were analysed with GraphPad Prism, version 5.

Supplementary references

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Supplemental table and figures

Table S7.1 Macrophage-, collagen-, lipid- and smooth muscle cell scores for the stable, intermediary and rupture-prone plaques of the Athero-Express.

	Plaque phenotype		
	Stable (n=26)	Intermediary (n=29)	Rupture-prone (n=20)
Macrophage scores			
0 (No staining), %	19.2	10.3	0
1 (Minor staining), %	80.8	24.1	0
2 (Moderate staining), %	0	48.3	60.0
3 (Heavy staining), %	0	17.2	40.0
Average score	0.8	1.7	2.4
Collagen scores			
0 (no collagen), %	0	0	40
1 (minor collagen), %	0	17.2	60
2 (moderate collagen), %	37.5	48.3	0
3 (heavy collagen), %	62.5	34.5	0
Average score	2.6	2.2	1.6
Lipid scores			
0 (no lipids), %	76.9	10.3	0
1 (<40% lipids), %	23.1	75.3	15.0
2 (>40% lipids), %	0	13.8	85.0
Average score	0.2	1.0	1.8
Smooth muscle scores			
0 (No staining), %	0	0	5
1 (Minor staining), %	0	27.6	95
2 (Moderate staining), %	46.2	48.3	0
3 (Heavy staining), %	53.8	24.1	0
Average score	2.5	2.0	1.0

The carotid atherosclerotic fragments were dissected into 0.5-cm-thick cross-sectional segments along the longitudinal axis of the vessel. The plaque segment showing the largest plaque burden was used for histological analysis and staining (H&E, Elastin von Gieson, picrosirius red, [alpha]-actin, and CD68). Subsequently, plaques of the atheromatous phenotype (collagen score 0 and 1; lipid score 2), rich in macrophages (score 2 and 3) and with low smooth muscle cell scores (0 and 1) were classified as rupture-prone plaques (n=20). Plaques with the fibrous phenotype (collagen 2 and 3; lipid score 0 and 1), low macrophage scores (0 and 1), and high smooth muscle cell scores (2 and 3) were characterized as stable (n=26). Plaques not falling in either category were classified as intermediate plaques (n=29).

Figure S7.1 Levels of MG-H1(A) and CML (B) in relation to plaque characteristics. Lysine-adjusted levels of MG-H1 (A) and CML (B) were measured in plaque homogenates with UPLC-MSMS and compared across straining categories of respectively plaque lipid content, collagen, smooth muscle cells, macrophages and overall lesion type, quantified in adjacent tissue. Data are expressed as geometric mean and 95% confidence interval. Statistical testing was performed for trend, using ANCOVA. Skewed variables were log-transformed. Analyses were adjusted for age, sex, smoking and presence of diabetes.

Figure S7.2 CML and MG-H1 co-localize with hypoxic regions and to a lesser extent with nitrotyrosine. Plaques of patients infused with hypoxia marker pimonidazole were stained for pimonidazole, CML, MG-H1 and nitrotyrosine.

Figure S7.3 Anoxia inhibits GLO1 activity and induces MGO and GO in U937 monocytes. U937 cells were cultured in anoxia (0% O₂) for 24 hours. GLO1 activity (A) was measured. MGO (B) and GO (C) were measured in the culture supernatant with UPLC-MSMS. Data are presented as mean and standard error. Differences were tested with student T-test for two independent samples.

Figure S7.4 TNF decreased GLO1 and increased MGO, and viability is reduced after incubation with MGO in murine macrophages. Murine macrophages were differentiated from bone-marrow of 10 week old C57Bl6 mice, and stimulated for 24 hours with mouse TNF (500U/ml). GLO1 activity (A) was measured with an activity assay. MGO (B) and GO (C) were measured in the culture supernatant with UPLC-MS/MS. In addition, the macrophages were stimulated for 24 hours with MGO (0.1, 0.2 and 0.4mmol/l) and viability was measured with a MTT assay (D). Propidium-Iodine FACS (E) was used to quantify cell death (SubG1 peak (F)) and proliferation (S-peak (G) and G2/M peak (H)). Data are presented as mean and standard error. Differences were tested with student T-test for two independent samples (A-C), and with ANOVA (D-I).

Figure S7.5 Co-incubation of U937 monocytes with TNF and NAC does not restore GLO1 or reduce MGO. U937 monocytes were incubated for 24 hours with TNF (100U/ml) and with NAC (1 mmol/l). GLO1 activity (**A**) was measured. MGO (**B**) and GO (**C**) were measured in the culture supernatant with UPLC-MS/MS. CML (**D**), quantification (**E**) was measured with Western-blot in the cell-lysate. Re-probing with β -actin ensured equal loading. Data are presented as mean and standard error. Differences were tested with ANOVA.

Figure S7.6 Increasing oxidative stress with H₂O₂ leads to a strong increase of CML in U937 monocytes. GLO-1 activity of monocytes incubated with 0, 1 or 10 μmol/l H₂O₂ was determined (A). Levels of MGO (B) and GO (C) were measured by UPLC-MS/MS. CML (D), quantification (E) was measured with Western-blot in the cell-lysate. Re-probing with β-actin ensured equal loading. Data are presented as mean and standard error. Differences were tested with ANOVA.

Figure S7.7 High glucose concentration does not decrease GLO1 or induce MGO or AGEs in U937 monocytes. U937 monocytes were cultured for 14 days in 30mmol/l high glucose (HG), or in mannitol (M) as an osmotic control. Oxidative stress was measured with CM-H₂DCFDA FACS (A) GLO1 activity (B) was measured. MGO (C) was measured in the culture supernatant with UPLC-MS/MS. CML (D), CEL (E) and MG-H1 (F) were measured with UPLC-MS/MS in the cell-lysate. Data are presented as mean and standard error. Differences were tested with student T-test for two independent samples.

Figure S7.8 Incubation of U937 monocytes with MGO induces formation of MG-H1 and CML in the cell-lysate. U937 monocytes were incubated for 24 hours with 0.1 mmol/l MGO, and MG-H1 (A), quantification in (B) and CML (C), quantification in (D) were detected with western blot in the cell-lysate. Re-probing with β -actin ensured equal loading. Data are presented as mean and standard error. Differences were tested with student T-test for two independent samples.

Chapter 8

**Glyoxalase 1 overexpression does not affect
atherosclerotic lesion size and severity in ApoE^{-/-}
mice with or without diabetes**

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Abstract

Objective

Advanced glycation endproducts (AGEs) and their precursors have been associated with the development of atherosclerosis. We recently discovered that glyoxalase 1 (GLO1), the major detoxifying enzyme for AGE-precursors, is decreased in ruptured human plaques and that levels of AGEs are higher in rupture-prone plaques. We here investigated whether overexpression of human GLO1 in ApoE^{-/-} mice could reduce development of atherosclerosis.

Methods and Results

We crossed C57BL/6 ApoE^{-/-} mice with C57BL/6 GLO1 overexpressing mice (huGLO1^{+/+}) to generate ApoE^{-/-} (n=16) and ApoE^{-/-} huGLO1^{+/+} (n=20) mice. To induce diabetes, we injected a subset with streptozotocin to generate diabetic ApoE^{-/-} (n=8) and ApoE^{-/-} huGLO1^{+/+} (n=13) mice. All mice were fed chow and sacrificed at 25 weeks of age.

The GLO1 activity was threefold increased in huGLO1^{+/+} aorta, but aortic root lesion size and phenotype did not differ between mice with- and without huGLO1^{+/+} overexpression. We detected no differences in gene expression in aortic arches, in AGE levels and cytokines, in circulating cells and endothelial function between ApoE^{-/-} mice with- and without huGLO1^{+/+} overexpression. Although diabetic mice showed decreased GLO1 expression ($P<0.05$) and increased lesion size ($P<0.05$) in comparison to non-diabetic mice, GLO1 overexpression also did not affect the aortic root lesion size or inflammation in diabetic mice.

Conclusion

In ApoE^{-/-} mice with or without diabetes, GLO1 overexpression did not lead to decreased atherosclerotic lesion size or systemic inflammation. Increasing GLO1 levels does not seem to be an effective strategy to reduce glycation in atherosclerotic lesions, likely due to increased AGE formation through GLO1 independent mechanisms.

Introduction

Rupture of an atherosclerotic plaque and subsequent thrombosis is the major cause of cardiovascular events such as myocardial infarction and stroke.^{1,2} Rupture-prone plaques are characterized by inflammation, macrophage infiltration and a large necrotic core.³ Inflammation of the plaque is characterized by high metabolic activity in macrophages, as characterized by high ¹⁸Fluor-deoxyglucose uptake.⁴ Advanced glycation endproducts (AGEs) are a large family of extensively sugar-modified proteins which are formed in plaques as a consequence of increased metabolic activity.⁵ We have recently shown that AGE levels are higher in rupture-prone than in stable human plaques.⁶ In addition, animal studies have shown that compounds decreasing AGEs, such as aminoguanidine, pyridoxamine and alagebrium attenuate atherosclerosis.⁷⁻⁹ Intracellular sugars and their derivatives, products of glycolysis, are potent precursors of AGE formation, with the dicarbonyl methylglyoxal (MGO) as the most reactive AGE precursor.¹⁰ MGO reacts primarily with arginine residues to form the AGE 5-hydro-5-methylimidazolone (MG-H1). We have recently shown that MG-H1 is increased in rupture-prone plaques,⁶ and that MGO-derived AGEs may explain the association between inflammation and growth of the necrotic core in advanced atherosclerosis.

MGO can be detoxified by glyoxalase 1 (GLO1), GLO2 and reduced glutathione into D-lactate,¹¹ thereby preventing accumulation of MGO and MGO-derived AGEs. As such, the glyoxalase pathway may be one of the major defence mechanisms against glycation from glycolysis *in vivo*. Indeed, GLO1 overexpression in rats reduces the accumulation of AGEs, endothelial dysfunction and diabetic micro-vascular complications.¹²⁻¹⁴ We have shown that GLO1 expression is decreased in ruptured plaques,⁶ with inflammation and hypoxia in plaques as potential determinants of reduced GLO1 activity and the increased formation of AGEs. However, as AGEs in the plaque may also be derived from other sources than intracellular sugars, such as lipid oxidation,¹⁵ an investigation into a causal role for GLO1 in atherosclerosis is needed.

We hypothesized that GLO1 plays a major protective role against developing plaque-rupture. To test this hypothesis, we have investigated whether human GLO1 overexpression (huGLO1^{+/+}) in ApoE^{-/-} mice reduces atherosclerotic lesion size, and induces a stable lesion phenotype.

Methods

GLO1 overexpressing ApoE^{-/-} Mice

The effect of GLO1 overexpression on atherosclerosis was studied in C57BL/6 ApoE^{-/-} mice (002052, JAX[®] Mice, Maine, USA) crossed with heterozygous C57BL/6 mice overexpressing ubiquitously human GLO1 (referred to as huGLO1^{+/+} mice). Littermates

were used as respective controls ($n=16$) for the ApoE^{-/-} huGLO1^{+/-} mice ($n=20$). For phenotyping of the backcrossed model, we also included a subset of wildtype control (WT, $n=5$) and wildtype huGLO1^{+/-} mice (WT huGLO1^{+/-}, $n=5$). We used males only. The generation of the ubiquitous human GLO1 overexpressing mouse has been described in detail previously.¹⁶ In short, the entire coding sequence of human GLO1 cDNA was cloned into the EcoR I site of the pBsCAG-2. The GLO1 transgene isolated by digestion of pBsCAG-2 containing GLO1 cDNA with Kpn I and Sac I was microinjected into one pronucleus of fertilized C57BL/6 eggs. Mice were housed under standard conditions and given free access to a normal chow diet and water.

At 25 weeks of age, the mice were killed by means of CO₂ inhalation, and subsequently exsanguinated by cardiac puncture. Experiments were performed according to Dutch laws, approved by the Committee for Animal Welfare of Maastricht University.

Streptozotocin-injected mice

To investigate whether GLO1 protects against increased atherogenesis in diabetes, we performed additional experiments with a subset of streptozotocin (STZ)-injected ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice. At 9 weeks of age, mice were injected with 50mg/kg STZ on 5 subsequent days to induce diabetes. We included mice that had fasting plasma glucose levels of >15mmol/l at 24 weeks of age (ApoE^{-/-} $n=8$; ApoE^{-/-} huGLO1^{+/-} $n=13$).

Blood lipids, insulin and leptin levels

At 24 weeks of age, mice were fasted for 6 hours to measure plasma cholesterol and plasma triglycerides using colorimetric methods, according to manufacturers (Instruchemie, Delfzijl, The Netherlands) protocol. Plasma insulin and leptin levels were measured using Meso Scale Discovery (Gaithersburg, USA) multispot Mouse cytokine assays for plasma, according to the manufacturer's instruction.

Plasma MGO, GO and 3-DG

Plasma levels of the major AGE precursors MGO, glyoxal (GO) and 3-deoxyglucosone (3-DG) were measured as described previously with ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MSMS).¹⁷ Intra-assay variations for MGO, GO and 3-DG were 2.9, 4.3 and 2.4%, respectively. All samples were measured in a single run, eliminating inter-run variation.

Plasma and aortic arch AGE levels

The aorta was carefully removed and cleaned of adherent tissue. Aortic arches were snap-frozen after careful dissection. Subsequently, mRNA and protein were extracted

using Trizol and a mini beat beater. Protein-bound N^ε-(carboxymethyl)lysine CML, and MGO-derived AGEs N^ε-(carboxyethyl)lysine (CEL) and MG-H1 were measured in the protein fraction of the homogenates of the aortic arch and in plasma using UPLC-MSMS by a single-run method described earlier.¹⁸ Intra-assay variation for CML, CEL and MG-H1 was 4.8, 5.0 and 18.1%, respectively. All samples were measured in a single run, eliminating inter-run variation.

Analyses of aortic roots

Aortic roots were fixed in 4% formaldehyde and subsequently embedded in paraffin. Consecutive 4 μm sections of the heart in the atrioventricular valve region were collected and stained with hematoxylin and eosin. For morphometric analysis, lesion size was measured on 5 consecutive sections at 24 μm intervals.

Quantification was performed just above the aortic cusps, at the start of the ascending aorta, as identified by the medial layer of smooth muscle cells and the presence of coronary orifices. In an additional analysis, we adjusted lesion size for aortic circumference by multiplying the lesion size by the ratio of the aortic circumference of each mouse divided by the average aortic circumference of mice (measured with Image J). As mice were not perfused prior to excision of the heart, care was taken that any remaining blood clots in the lumen were excluded from analysis. In addition, we performed a Sirius red staining for collagen deposition in the plaque. Using brightfield and polarized light microscopy, we investigated the spectrum of thick (red) to thin (green) collagen.¹⁹ Histological analyses were performed in a blinded manner using Leica QWin3 quantification software (Leica, Wetzlar, Germany). Lesions were also phenotyped according to lesion severity (early, moderate and severe), as described earlier,²⁰ and semi-quantitatively scored by an experienced mouse pathologist, in a blinded manner, for amount of foam cells, small macrophages, monocyte adhesion, granulocytes, adventitial influx and apoptosis ranging from 0 (absent) to 4 (abundant). In a random subset of animals (*n*=5 per group) we stained aortic roots for GLO1 to confirm overexpression.

mRNA analyses of aortic arches

qPCR analyses were performed for oxidative stress reducing enzymes (nuclear factor erythroid 2-related factor 2 (NRF2), Kelch-like ECH-associated protein 1 (Keap1), NAD(P)H dehydrogenase (quinone1) (NQO1), Glutathione peroxidase 1 (Gpx1), catalase (CAT) and heme oxygenase 1 (HMOX1)), macrophage marker F4/80 ratio, inflammatory markers interleukin 6 (IL-6), monocyte chemotactic protein-1 (MCP-1), and for mouse and human GLO1 to confirm overexpression in the aortic arch. Gene-expressions were adjusted for Beta-2-microglobulin and cyclophilin A as housekeeping genes.

GLO1 activity in thoracic aorta

A segment of the descending thoracic aorta, directly below the aortic arch, was stored for GLO1 activity measurement. Protein was extracted in GLO1 assay buffer using a mini-beat beater. GLO1 activity was measured by the method of McLellan et al., and expressed as units per mg protein.²¹ To obtain sufficient protein, three aortas were pooled for each measurement.

Murine macrophages

Bone marrow-derived murine macrophages were obtained by differentiating femoral and tibial bone marrow suspensions as described previously,²² to confirm overexpression in macrophages at activity level and to investigate the effect of oxidized lipids on GLO1 activity and AGE precursor formation. At day 9, the macrophages were plated at 0.5×10^6 cells/ml, incubated with copper oxidized LDL (ox-LDL) for 25 μ g/ml ox-LDL for 16 hours, and harvested to measure GLO1 activity, the cell culture supernatant was stored for measuring AGE-precursors.

FACS analyses

At 24 weeks of age, in a subset of the animals (ApoE^{-/-}, $n=7$; ApoE^{-/-} huGLO1^{+/-}, $n=9$), using Flow cytometry, we measured cell counts of circulating monocytes, of both the Ly6C^{high} and Ly6C^{low} subset, CD4⁺ T-cells, CD-8⁺ T-cells and B-cells. Cells were stained with anti- CD45-PerCP, NK1-PE, CD3-Pacific Blue, CD8-FITC, CD4-APC-H7, Ly6G-APC-H7, Ly6C-APC, B220-V500 and CD11b-PE-cy7. Cells were analysed using a FACS-Canto II (BD Biosciences).

Plasma cytokines

Plasma levels of MCP-1, interferon gamma (IFN), interleukin (IL)-10, IL-12, IL-1 β , IL-6 and mCK were measured using Meso Scale Discovery (Gaithersburg, USA) multispot Mouse cytokine assays for plasma, according to the manufacturer's instruction. Intra- and inter-assay coefficient of variation were, respectively, for MCP-1 (3.1 and 4.0%), IL-10 (5.2 and 22.8%), IL-1 β (16.9 and 26.5%), IL-6 (6.9 and 2.8%), mKC (4.5 and 20.4%) and IFN- γ (6.2 and 10.9%).

Vascular reactivity of aortic segments

After careful dissection of the aorta, following the description by Crauwels et al.,²³ the atherosclerosis-prone aortic segment of the descending thoracic aorta was excised just above the diaphragm. One segment was mounted in a myograph organ bath (model 610M Danish Myotechnology by J.P. Trading, Denmark) with two steel 40- μ m wires inserted through the lumen of the segments. The organ bath contained fresh Krebs-Ringer bicarbonate solution (KRB) containing 118 mM NaCl; 4.7 mM KCl;

1.2 mM KH₂PO₄; 25 mM NaHCO₃; 1.1mM MgSO₄; 2.5 mM CaCl₂ and 5.0mM glucose, maintained at 37°C and gassed continuously with 95% O₂ and 5% CO₂ (pH 7.4). The internal diameter of each vessel was normalized by stretching the vessel to a diameter that yielded a wall tension equivalent to a transmural pressure of 100mmHg. The isometric tension generated by the vessels was recorded using Powerlab 4/25 (ADInstruments) connected to the Myo-Interface.

Maximum contraction was measured by incubation with 125 mM K⁺ plus 10μM phenylephrine (Sigma-Aldrich). Endothelium-dependent vasorelaxation was tested with cumulative concentrations of acetylcholine (ACh, Sigma-Aldrich, 0.001–10 μM) during contraction induced by 10μM phenylephrine, in the absence and presence of 10 μM indomethacin (Sigma-Aldrich) to block the synthesis of prostaglandins. Nitric oxide (NO)-independent relaxation was tested during contraction with 10 μM phenylephrine in the presence of 100 μM L-NAME (Sigma-Aldrich) to block NO synthases. Endothelium-independent relaxation was tested with the NO-donor sodium nitroprusside (SNP, Sigma-Aldrich, 0.001–10μM) during phenylephrine-induced contraction (10 μM). We used male C57BL/6 mice to determine a reference-curve.

Statistical analyses

Data are presented as mean +/- standard error of the mean (SEM). When appropriate, variables were log-transformed prior to analysis to reduce influence of outliers. Characteristics of mice were compared with ANOVA with Bonferroni correction when comparing 4 groups and Students T-test when comparing 2 groups. Lesion severity and semi-quantitative scoring was tested with a chi-square test.

In addition, to analyse the vascular reactivity, sensitivity (pD₂) and efficacy (E_{max}) was calculated from the concentration–response curves to ACh. pD₂ is defined as the negative logarithm to base 10 of the EC50 values and E_{max} is defined as the maximal vasorelaxation to ACh at the highest concentration used and expressed as 100 minus percentage relative contractions to 10μM phenylephrine. The concentration-response curves were compared using repeated measurements analyses of variance (ANOVA). As Mauchly's test for sphericity was significant (*P*<0.05), these analyses were Greenhouse-Geisser corrected. Data were analysed using SPSS, version 20. For all analyses, a p-value of <0.05 was considered statistically significant.

Results

Confirmation of GLO1 overexpression

In line with our previous work in humans, in ApoE^{-/-} mice we found that GLO1 staining was predominantly present in cytoplasm of cells in the fibrous cap and macrophage rich regions, while we found very little staining in and around the necrotic core (Figure

8.1A,B). We did find high expression of the human GLO1 mRNA in the aortic arches of ApoE^{-/-} huGLO1^{+/-} mice, while mouse GLO1 expression was not changed in huGLO1^{+/-} mice (Figure 8.1C,D). Immunohistochemistry confirmed increased GLO1 staining in huGLO1^{+/-} mice, with a striking increase in the heart (Figure 8.1A,B arrow 1). GLO1 activity in the thoracic aorta was approximately 3-fold increased in huGLO1^{+/-} mice (Figure 8.1E). In line, in bone marrow derived macrophages of the ApoE^{-/-} huGLO1^{+/-} mice GLO1 activity was increased by approximately 4-fold compared to ApoE^{-/-} (Figure 8.1F). In comparison to WT mice, both endogenous GLO1 and transgenic human GLO1 mRNA were higher, while GLO2 mRNA levels were lower in ApoE^{-/-} than in WT mice (Figure S8.1A-C). However, we did not find any difference in GLO1 activity between WT and WT huGLO1^{+/-} or ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice (Figure S8.1D).

Figure 8.1 Confirmation of GLO1 overexpression in the ApoE^{-/-} huGLO1^{+/-} mice. Sections of the heart in the atrioventricular valve region were stained with anti GLO1. Arrow 1 indicates myocardial tissue; arrow 2 indicates macrophages and arrow 3 indicates aortic media. (A and B, representative pictures, 50x enlargement). Mouse GLO1 (C) and human GLO1 (D) mRNA were measured in aortic arch of the ApoE^{-/-} (n=16) and the ApoE^{-/-} huGLO1^{+/-} (n=20) mice to confirm expression of the construct. GLO1 activity was measured in thoracic arches to confirm vascular overexpression at activity level (E). Bone marrow derived macrophages were generated from ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice and GLO1 activity was measured to confirm overexpression in the macrophage (F). Data are shown as mean ± S.E.M. Differences were tested with students T-test.

General characteristics

The characteristics of the mice are listed in Table 8.1. Weight, fasting plasma glucose, total cholesterol, triglycerides, insulin and leptin did not differ significantly between ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice.

Plasma and aortic arch AGE levels

Surprisingly, we found no differences of plasma levels of MGO, GO and 3-DG (Table 8.1). Furthermore, the levels of CML, CEL and MG-H1 in plasma and aortic arch did not differ between mice with- and without GLO1 overexpression (Table 8.1).

Table 8.1 Characteristics of the mice at 25 weeks.

Characteristic	Euglycemic		Diabetic (STZ-injected)	
	ApoE ^{-/-}	ApoE ^{-/-} huGLO1 ^{+/-}	ApoE ^{-/-}	ApoE ^{-/-} huGLO1 ^{+/-}
Groupsize (n)	16	20	8	13
Weight (gram)	30.0 ± 0.6	29.9 ± 0.6	25.4 ± 1.0*	24.6 ± 0.8*
Fasting plasma glucose (mmol/l)	7.3 ± 0.3	6.3 ± 0.5	22.2 ± 2.4*	22.2 ± 1.7*
Fasting total cholesterol (mmol/l)	17.4 ± 1.2	15.9 ± 1.1	23.9 ± 2.4*	26.2 ± 1.7*
Fasting tryglicerids (mmol/l)	1.6 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
Urine production (ml/24 hours)	1.0 ± 0.1	1.0 ± 0.1	10.2 ± 2.7*	6.8 ± 1.9*
Plasma insulin	700.0 ± 108.3	674.1 ± 135.8	263.0 ± 106.3	679.7 ± 273.8
Plasma leptin	1246.3 ± 150.0	913.1 ± 87.5	174.1 ± 39.2*	297.1 ± 67.1*
Plasma AGE precursors				
MGO (nmol/l)	661.95 ± 67.73	721.11 ± 107.03	858.5 ± 101.0	824.1 ± 58.8
GO (nmol/l)	1083.27 ± 50.03	1078.22 ± 65.36	1777.6 ± 165.5*	1602.8 ± 88.1*
3-DG (nmol/l)	2346.76 ± 107.39	2243.61 ± 90.48	4345.6 ± 202.6*	4330.3 ± 212.9*
Plasma AGEs				
CML (nmol/mmol lysine)	30.4 ± 0.9	30.6 ± 1.2	40.1 ± 1.5*	40.9 ± 1.7*
CEL (nmol/mmol lysine)	11.2 ± 0.7	11.6 ± 0.8	8.5 ± 0.6	8.5 ± 0.8
MG-H1 (nmol/mmol lysine)	101.0 ± 8.1	106.2 ± 4.6	91.0 ± 6.0	112.6 ± 17.5
Aortic arch AGEs				
CML (nmol/mmol lysine)	82.46 ± 3.36	86.84 ± 4.14	109.1 ± 10.2*	104.3 ± 8.6
CEL (nmol/mmol lysine)	56.13 ± 3.58	58.62 ± 2.87	62.2 ± 6.4	62.0 ± 5.8
MG-H1 (nmol/mmol lysine)	58.77 ± 4.97	77.56 ± 5.33	71.0 ± 9.0	78.2 ± 8.4

Data are shown as mean ± S.E.M. Differences tested with ANOVA and Bonferroni correction. *P<0.05 relative to non-diabetic ApoE^{-/-} mice.

Lesion size and severity of the aortic root

GLO1 overexpression did not reduce lesion size (Figure 8.2A-B) and percentage of necrotic core (Figure 8.2C) or cholesterol clefts (Figure 8.2D). Adjusting lesion size for aortic circumference did not change the results (Figure S8.2A). In addition, lesion severity was comparable between the groups (Figure 8.2E). We also found no difference in plaque collagen content (Figure 8.1F-I). Further analysis and scoring of plaque morphology revealed no difference in foam cells (Figure S8.3A), macrophages (Figure S8.3B), monocytic adhesion (Figure S8.3C), granulocytes (Figure S8.3D),

adventitial influx (Figure S8.3E) or apoptotic morphology (Figure S8.3F). These results were similar when we analyzed early, moderate and advanced plaques separately.

Figure 8.2 Lesion size, cholesterol clefts, necrotic core size and lesion severity of the aortic root did not differ between ApoE^{-/-} mice with- and without GLO1 overexpression. Consecutive 4 μm sections of the hearts of the ApoE^{-/-} (n=16) and ApoE^{-/-} huGLO1^{+/-} (n=20) in the atrioventricular valve region were collected and stained with hematoxylin and eosin (A), 50x enlargement). Morphometric analysis of lesion size (B), cholesterol clefts (C), and necrotic core size (D) was measured on 5 consecutive sections in 24 μm intervals. (E) Shows the categories of lesion severity (early, moderate and advanced), differences were tested by chi-square test. Next, using Sirius red (brightfield (F), polarized light (H), 200x enlargement), we quantified thick (red, (G) and thin (green, (I)) collagen. Data are shown as mean ± S.E.M. Differences were tested with students T-test.

mRNA expression oxidative stress markers aortic arches

Gene expression of the antioxidant markers NFR2 (Figure S8.4A), Keap1 (Figure S8.4B), NQO1 (Figure S8.4C), GPx1 (Figure S8.4D) were decreased, while HMOX1 (Figure S8.4F) was increased in aortic arches ApoE^{-/-} mice, indicating high levels of

oxidative stress in the ApoE^{-/-} mice, and this was not improved in the ApoE^{-/-} huGLO1^{+/-} mice.

mRNA expression inflammatory markers aortic arches

Gene expression of F4/80 in aortic arches did not differ between mice with- and without GLO1 overexpression (Figure S8.5A), indicating similar macrophage content. AGEs are a ligand for RAGE, an important activator of NF- κ B,²⁴ but mRNA expression of NF- κ B regulated genes such as IL-6 (Figure S8.5B) and MCP-1 (Figure S8.5C) revealed no differences between the groups, indicating that plaque inflammation was similar.

Plasma cytokines and circulating cells

We also determined the effect of GLO1 overexpression on systemic inflammation by measuring plasma cytokines and circulating cells. Plasma levels of MCP-1, IL-10, IL-1 β , IL-6, mKC and IFN- γ (Figure 8.3A-F) did not significantly differ between animals with- or without GLO1 overexpression. In line, we did not observe any effect of GLO1 overexpression on circulating CD4⁺ T-cells (Figure S8.6B), CD-8⁺ T-cells (Figure S8.6C), B-cells (Figure S8.6D) and monocytes (Figure S8.6E), nor on both the Ly6C^{high} (Figure S8.6F) and Ly6C^{low} (Figure S8.6G) subsets.

Figure 8.3 Plasma cytokine levels did not differ between ApoE^{-/-} mice with- and without GLO1 overexpression. Levels of MCP-1 (A), IL-10 (B), IL-1 β (C), IL-6 (D), mKC (E) and IFN- γ (F) were measured in plasma of the ApoE^{-/-} ($n=16$) and ApoE^{-/-} huGLO1^{+/-} mice ($n=20$) using multispot mouse cytokine array. Data are shown as mean $\pm$ S.E.M. Differences were tested with students T-test.

Vascular reactivity of aortic segments

As we also observed accumulation of AGEs in endothelial cells of atherosclerotic plaques,⁶ we determined the vascular function by measuring ex-vivo the endothelium-dependent and -independent vaso-relaxation in atherosclerosis-prone aorta segments. Aortic diameter in ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} (801±18 and 768±24 µm, respectively) and maximum contraction did not differ between the two groups (active wall tension in ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} was 1.88±0.2 and 1.53±0.2 N/m, respectively). Under baseline conditions, high concentrations of ACh evoked an endothelium-dependent contraction, which was not significantly different between the two groups (Figure 8.4A). Inhibition of prostaglandin production by indomethacin abolished this response (Figure 8.4B). Under these conditions the total dose response curve to ACh was not statistically different between the ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice. However, both ApoE^{-/-} genotypes showed strong impairment compared with an aorta of the C57BL/6 wild-type control mouse. Also, NO-independent (Figure 8.4C) and endothelium-independent (Figure 8.4D) relaxation did not differ significantly between the ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice concerning the shift of the curve. Additional analysis showed that the efficacy (E_{max}) was mildly, but significantly, decreased in the ApoE^{-/-} huGLO1^{+/-} compared to the ApoE^{-/-} mice (Figure 8.4B and 8.4C, Table 8.2). The potency (pD₂ values) was mildly decreased in the ApoE^{-/-} huGLO1^{+/-} mice (Figure 8.4D).

Figure 8.4 GLO1 overexpression did not improve aortic endothelial reactivity. White and black circles represent ApoE^{-/-} (n=9) and ApoE^{-/-} huGLO1^{+/-} (n=7) mice respectively. Grey triangles represent the curves of a wild type C57BL/6 mouse to depict normal values. Carefully dissected aortic rings were mounted in a wire-myograph and acetylcholine (ACh) induced relaxation was investigated under basal conditions (A), in the presence of indomethacin (NO-mediated relaxation) (B), and in the presence of indomethacin and L-NAME (NO-independent relaxation) (C). Next, endothelium-independent, Na nitroprusside (SNP)-induced vasorelaxation was investigated (D). Data are presented as mean ± S.E.M. Differences between the curves were tested with repeated measurements ANOVA.

Table 8.2 Potency and efficacy of NO-mediated relaxation, NO-independent relaxation and SNP-induced relaxation of the thoracic aorta.

	ApoE ^{-/-}	ApoE ^{-/-} huGLO1 ^{+/-}
NO-mediated relaxation		
Potency (pD ₂)	6.90 ± 0.34	6.79 ± 0.46
Efficacy (E _{max}) (%)	37.3 ± 5.1	21.1 ± 3.8 *
NO-independent relaxation		
Potency (pD ₂)	6.76 ± 0.17	6.69 ± 0.29
Efficacy (E _{max}) (%)	38.9 ± 2.5	20.1 ± 3.0 *
SNP-induced relaxation		
Potency (pD ₂)	8.28 ± 0.05	7.99 ± 0.07 *
Efficacy (E _{max}) (%)	98.0 ± 1.0	96.3 ± 1.6

* $P < 0.05$ compared with ApoE^{-/-} (students T-test).

Incubation of murine macrophages with oxidized LDL

When we incubated murine macrophages derived from ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice (Figure S8.7A) with ox-LDL, we observed in the supernatant increased 3-DG levels (Figure S8.7B), a trend for increased MGO levels, but this was not significant (Figure S8.7C), and no change in GO levels (Figure S8.7D). Importantly, this was similar for both the macrophages derived from ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice.

Diabetic mice

Since diabetes strongly increases atherosclerosis, and we previously found increased levels of AGEs and their precursors in diabetic rats, which could be reduced by GLO1 overexpression,²⁵ we performed an additional series of experiments with STZ-injected, diabetic ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice. Interestingly, expression of mouse GLO1 was approximately 40% lower in the diabetic mice (Figure 8.5A). Furthermore, in the diabetic mice, we observed a significant increase of CML and particularly GO, while MGO was only mildly increased (Table 8.1). However, these compounds were not significantly reduced by GLO1 overexpression (Table 8.1). Although diabetes also increased lesion size compared to non-diabetic mice (Figure 8.5B), GLO1 overexpression did not decrease plaque lesion size (Figure 8.5C). Adjusting lesion size for aortic circumference did not change the results (Figure S8.2B). Furthermore, plasma MCP-1 (Figure 8.5D), F4/80 (Figure 8.5E) and MCP-1 (Figure 8.5F) expression in the aortic arch or circulating monocytes (Figure 8.5G) of the diabetic mice were not changed by GLO1 overexpression.

Figure 8.5 GLO1 overexpression did not decrease lesion size in diabetic ApoE^{-/-} mice. Mice were injected with STZ to induce diabetes (A, B), ApoE^{-/-} ($n=8$) and ApoE^{-/-} huGLO1^{+/-} ($n=13$) mice were combined, to compare euglycemic and diabetic mice. We found that diabetes strongly impaired mouse GLO1 expression (A) and increased lesion size (B). Aortic root lesion size (C), plasma MCP-1 (D), F4/80 (E) and MCP-1 (F) aortic arch expression and circulating monocytes (G) did not differ between diabetic ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice. Data are shown as mean $\pm$ S.E.M. Differences were tested with students T-test.

Discussion

This study shows that ubiquitous GLO1 overexpression does not reduce atherosclerotic lesion size, lesion severity, lesion inflammation, plasma cytokines, circulating monocytes, or endothelial dysfunction in ApoE^{-/-} mice. While diabetes

strongly impaired mouse GLO1 expression and increased lesion size, increased GO and CML levels were not reduced by huGLO1 overexpression nor the atherosclerotic and inflammatory phenotype.

Although we previously found that GLO1 expression is reduced and AGEs are increased in human ruptured plaques,⁶ we did not find a reduction of plasma and aortic arch levels of the AGEs CML, CEL and MG-H1 in the ApoE^{-/-} huGLO1^{+/-} mice with a 3 to 4-fold increase of GLO1 activity in the aorta and macrophages. This is in contrast to our previous findings of reduced plasma and tissue AGEs in diabetic rats with GLO1 overexpression, in which we found a similar increase of GLO1 staining in GLO1 activity in the aorta as in the current model.²⁵ Furthermore, others have found that overexpression of GLO1 also reduces AGEs and diabetic complications such as nephropathy²⁶ and neuropathy²⁷ and improved recovery from hind limb ischaemia²⁸ in STZ-injected mice. However, others have recently also found that both GLO1 overexpression and knockdown does not reduce atherosclerosis in diabetic ApoE deficient mice.²⁹ The failure of GLO1 overexpression to reduce AGEs may therefore be limited to the setting of the ApoE^{-/-} mouse, although, a previous report showed also that upregulation of GLO1 failed to reduce MGO levels in diabetic mouse lenses.³⁰ Although we could argue that this discrepancy could be explained by the mouse GLO1 downregulation we found in the diabetic mice, this is unlikely, as the huGLO1^{+/-} overexpression was unaltered by diabetes (data not shown). Furthermore, it could be argued that we may have missed a subtle reduction of AGEs by huGLO1^{+/-} overexpression, this is also unlikely, as our intervention did not influence any of our other outcome parameters. As the ApoE^{-/-} mouse model is characterised by a profound dyslipidaemia (Table 1), and lipid oxidation has been shown to be an important source of AGEs,¹⁵ independently of glucose levels, it is more likely that huGLO1^{+/-} overexpression may not be protective against AGE-formation in hyperlipidaemia conditions such as in ApoE^{-/-} mice. We postulate that GLO1 may not come into sufficient contact with the AGE precursors derived from lipid oxidation. This may be likely, as GLO1 is present in the cytosol, where glycolysis takes place, but is not present in the cellular membranes and lipid particles where lipid oxidation largely takes place.³¹ This is further substantiated by our current study, as we could not detect any extracellular staining for GLO1 (Fig 1A,B) and we showed that in diabetic ApoE^{-/-} mice both glucose and cholesterol levels are elevated, while GO and CML, which are also derived from lipids,¹⁵ increased much more than MGO, which is mainly derived from glucose metabolism. In addition, ox-LDL increased MGO and 3-DG in cultured macrophages derived from both ApoE^{-/-} and ApoE^{-/-} huGLO1^{+/-} mice, supporting the notion that oxidized lipids indeed may contribute to GLO1 independent AGE formation. These results are in line with human data where no associations between plasma glucose and plaque AGEs levels were found, while plaque lipid content was associated with increased plaque AGEs.⁶ Also, in humans, statins which reduce cholesterol, also reduce plaque AGEs and risk of CVD,³² while

intensive glucose lowering treatment does not consistently lower risk of CVD.³³ These studies strongly suggest that in atherosclerotic plaques, lipid metabolism may serve as a more important source for AGEs than glucose and that huGLO1^{+/-} overexpression is not able to rescue this pathway. This is an important finding that should be further addressed in mechanistic studies.

We found that diabetes strongly impaired vascular mouse GLO1 expression, which is in line with a previous finding in peripheral nerves.²⁷ Whether this downregulation is directly attributable to high glucose levels is uncertain, as diabetes is also associated with more inflammation and advanced atherosclerosis, factors which are associated with a decrease of GLO1.⁶ Furthermore, despite increased GLO1 activity in the aorta, measured with the GLO1 assay, the *in vivo* activity of GLO1 may be hampered by low availability of reduced glutathione. Atherosclerosis is characterized by high oxidative stress,³⁴ as is also demonstrated by the altered expression of antioxidant genes we found in the aortic arch (Figure S8.3). Importantly, this was not improved by GLO1 overexpression. This state of oxidative stress in turn may deplete glutathione, which in its reduced form is needed to convert MGO into D-lactate by the glyoxalase system. This may be further aggravated by the decreased GLO2 expression, which may lead to a further reduction of glutathione recycling, halting the propagation of the glyoxalase pathway. Conversely, oxidised glutathione has been demonstrated to be a strong inhibitor of GLO1 activity.³⁵ In addition, GLO1 activity may be further reduced in the plaque by nitric oxide,³⁶ which is amply produced by inflammatory macrophages. Indeed, we showed that inflammation and hypoxia, hallmarks of advanced atherosclerosis, are potent inhibitors of GLO1 activity.⁶ Whether a depletion of glutathione, needed in catalytic concentrations by the glyoxalase system, leads to decreased glyoxalase activity in ApoE^{-/-} mice should be addressed in a future study. The glyoxalase system plays a vital role in maintaining cellular physiology, as it is found in organisms ranging from bacteria,³⁷ plants³⁸ to humans. Moreover, Bierhaus et al. proved that GLO1 deficient mice are not viable.²⁷ In fact, recent work of Tikellis et al. demonstrated that mice treated with a GLO1 inhibitor indeed displayed increased atherosclerosis, suggesting that strongly reduced GLO1 activity, far below physiological levels, indeed impairs vascular function and increases atherosclerosis. However, this finding is in contrast with a recent report that GLO1 knockdown does not increase atherosclerosis in ApoE deficient mice.³⁹ We therefore cannot yet conclude that GLO1 plays no role in the vascular physiology of the ApoE^{-/-} mouse. However, our data shows that simply increasing GLO1 expression levels and activity beyond those of control levels is insufficient to reduce glycation and atherosclerosis in ApoE deficient mice. Lipid-derived and GLO1 insensitive formation of AGEs or reduction of GLO1 activity by post-translational modification or depletion of glutathione *in vivo* may explain why we found no decrease in AGEs by the GLO1 overexpression in the current model.

Several experimental studies have suggested that AGEs play an important role in the development of atherosclerosis. Firstly, it has been shown that several AGE-lowering compounds such as aminoguanidine, pyridoxamine and alagebrium reduce atherosclerosis in ApoE^{-/-} mice.^{8,9} However, it should be noted that these compounds also exert anti-oxidant effects, and may thus partly attenuate atherosclerosis via AGE-independent mechanisms. Secondly, other enzymatic systems, such as aldose reductase are also known to detoxify MGO. Indeed, aldose reductase ApoE double knockout mice displayed increased plasma and plaque AGEs, and increased plaque burden.⁴⁰ Thirdly, in ApoE^{-/-} mice RAGE deletion greatly attenuates atherosclerosis development and induces a stable lesion phenotype,⁴¹ although we should note that RAGE is a multiligand receptor⁴² and disruption of inflammatory signalling by other RAGE ligands such as s100b and high motility group B1 may explain these findings. Therefore, additional studies designed to specifically reduce AGEs and MGO, without influencing other AGE-independent athero-protective pathways are still needed to investigate how large the role of AGEs really is in the development of atherosclerosis. To achieve this, the design of a more selective MGO-inhibiting molecule may be necessary.

In conclusion, our study suggests that increased protein levels of GLO1 alone is not sufficient to attenuate plasma and plaque AGEs and the development of atherosclerosis in ApoE^{-/-} mice. Therefore, additional experimental studies are needed to further unravel the complex role of the accumulation of AGEs, and MGO in particular, in atherosclerosis. Importantly, our study suggests that future strategies aimed at increasing GLO1 levels to reduce plaque AGEs may prove insufficient to treat cardiovascular disease.

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Supplemental figures

Figure S8.1 GLO1 activity in the aortic arch does not differ between wild type and ApoE^{-/-} mice. Human GLO1 (A) and Mouse GLO1 (B) and GLO2 (C) mRNA were measured in aortic arch of wildtype (WT, $n=5$), wildtype huGLO1^{+/+} (WT huGLO1^{+/+}, $n=5$) as well as ApoE^{-/-} ($n=16$) and ApoE^{-/-} huGLO1^{+/+} ($n=20$) mice to investigate whether expression of the construct and endogenous GLO1 and GLO2 were affected by the ApoE^{-/-} background. GLO1 activity was measured in thoracic arches to confirm vascular overexpression at activity level (D). Data are shown as mean ± S.E.M. Differences were tested with ANOVA and Bonferroni correction.

Figure S8.2 Adjusting lesion size for aortic diameter did not materially change the results in mice without (A) and with diabetes (B). Vessel circumference of the ascending aorta was measured with Image J. Lesion size was adjusted for aortic circumference by multiplying the lesion size by the ratio of the vessel diameter of each mouse divided by the average vessel diameter of all mice.

Figure S8.3 Histological scoring of aortic roots did not differ between mice with- and without GLO1 overexpression. Aortic roots of $ApoE^{-/-}$ ($n=16$) and $ApoE^{-/-}$ huGLO1 ($n=20$) mice were fixated in 4% formaldehyde and subsequently embedded in paraffin. Consecutive 4 μ m sections of the heart in the atrioventricular valve region were collected and stained with hematoxylin and eosin. Next, plaques were scored, ranging from 0 to 4, by an experienced mouse pathologist in a blinded manner. (A) Foam cells, (B) small macrophages, (C) monocyte adhesion, (D) granulocytes, (E) adventitial influx, (F) apoptosis. Data are shown as percentages per scoring category, and differences were tested with a Chi-square test.

Figure S8.4 mRNA expression levels of NRF2, Keap1, NQO1, GPx1 are decreased, while HMOX1 is increased in aortic arches ApoE^{-/-} mice with- and without huGLO1^{+/-} overexpression. Aortic arches of wildtype (WT, $n=5$), wildtype huGLO1^{+/-} (WT huGLO1^{+/-}, $n=5$) as well as ApoE^{-/-} ($n=16$) and ApoE^{-/-} huGLO1^{+/-} ($n=20$) were snap-frozen after careful dissection. Subsequently, mRNA was dissolved in Trizole using a mini beat beater. qPCR analyses were performed for NRF2 (A), Keap1 (B), NQO1 (C) and GPX1 (D), CAT (E), and HMOX1 (F). Data are shown as mean $\pm$ S.E.M. Differences were tested with ANOVA and Bonferonni correction.

Figure S8.5 mRNA expression levels of F4/80, IL-6 and MCP1 in aortic arches. Aortic arches of ApoE^{-/-} ($n=16$) and ApoE^{-/-} huGLO1^{+/-} ($n=20$) were snap-frozen after careful dissection. Subsequently, mRNA was dissolved in Trizole using a mini beat beater. qPCR analyses were performed for F4/80 (A), IL-6 (B) and MCP1 (C). Data are shown as mean $\pm$ S.E.M. Differences were tested with a T-test.

Figure S8.6 Circulating cells did not differ between ApoE^{-/-} mice with- and without GLO1 overexpression. (A) Shows representative FACS plots. Cell counts of circulating CD4⁺ T-cells (B), CD8⁺ T-cells (C), B-cells (D), monocytes (E), of the Ly6C^{high} (F) and Ly6C^{low} subset (G) were measured in a subset of the ApoE^{-/-} ($n=7$) and ApoE^{-/-} huGLO1^{+/-} ($n=9$) with flow cytometry. Data are shown as mean $\pm$ S.E.M. Differences were tested with students T-test.

Figure S8.7 Incubating bone marrow derived macrophages with ox-LDL mildly increases the AGE precursor 3-DG and mildly decreases GLO1 activity. Bone marrow derived macrophages, from ApoE^{-/-} mice (C) and ApoE^{-/-} huGLO1^{+/+} mice (GLO1) were differentiated and incubated with 25μg/ml ox-LDL for 24 hours. GLO1 activity (A) was measured and 3-DG (B), MGO (C) and GO (D) and were measured in the cell culture supernatant using UPLC-MSMS. Differences were tested with ANOVA and Bonferonni correction.

Chapter 9

Summary and main findings

Discussion

Valorisation

Summary and main findings

The rising prevalence of diabetes represents a global challenge. According to the International Diabetes Federation (IDF), a staggering 382 million people now have diabetes, and type 2 diabetes (T2DM) makes up the majority of cases. Cardiovascular diseases (CVD), such as heart attack and stroke, are major causes of mortality among individuals with T2DM. The process underlying the majority of cardiovascular events is rupture of an atherosclerotic plaque.¹ It is unknown why in some plaques fibrotic processes dominate, leading to stable plaques, while in other plaques inflammation and necrosis take over, leading to thinning of the fibrotic cap, presumably predisposing plaques to rupture.¹ Insight into this process could yield new treatments preventing plaque rupture, and could lead to new methods to identify people at increased risk of cardiovascular events at earlier stages.

It has been suggested that high metabolic activity in atherosclerotic plaques may contribute to the development of plaque rupture.² One consequence of increased metabolic activity is formation of advanced glycation endproducts (AGEs),³ and several studies have shown that AGEs accumulate in human atherosclerotic plaques.⁴⁻⁷ AGEs are a large family of extensively and irreversibly sugar-modified proteins, but rather than glucose itself, the toxic dicarbonyl compound methylglyoxal (MGO) has been identified as the most reactive AGE precursor.⁸ The glyoxalase system has been identified as a major pathway to detoxify MGO and prevent accumulation of AGEs.⁹

In this thesis it was hypothesized that accumulation of MGO and MGO-derived AGEs as well as dysfunction of the glyoxalase system are major factors contributing to CVD, especially in people with T2DM. We tested this hypothesis using data from large cohort studies, AGE measurements in human atherosclerotic plaques, *in vitro* studies and animal research. The main findings were:

- **Chapter 2.** Several surrogate markers were used in this thesis to measure atherosclerosis in large cohorts. For one of these, the ankle-brachial index (ABI), it was unknown whether it was a suitable marker for CVD in individuals with diabetes, as had already been demonstrated in individuals without diabetes. We found in the Hoorn study that the association between a low ABI and cardiovascular mortality did not differ between individuals with and without T2DM.
- **Chapter 3.** We developed a new technique to measure D-lactate, as a marker of MGO-detoxification, and found that D-lactate levels in plasma and urine were higher in individuals with T2DM.

- **Chapter 4.** We refined an existing (ultra-performance) liquid chromatography-tandem MS (UPLC-MS/MS) method to measure several protein-bound and free plasma AGEs. In a 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 AGEs (N^ε(carboxymethyl)lysine (CML), N^ε(carboxyethyl)lysine (CEL), pentosidine) and free AGEs (CML, CEL and 5-hydro-5-methylimidazolone (MG-H1)) with T2DM and prevalent CVD.
- **Chapter 5.** We found higher circulating GLO1 mRNA and plasma D-lactate levels in individuals with T2DM, but we did not find any associations among circulating GLO1 mRNA, plasma or urinary D-lactate and carotid intima media thickness, ABI and prevalent CVD.
- **Chapter 6.** We found that higher protein-bound plasma AGE levels were associated with a higher risk of incident CVD after adjustment for confounders, in a case-cohort study containing a subset of Dutch diabetic individuals from the European Prospective Investigation into Cancer and Nutrition (EPIC-NL).
- **Chapter 7.** We demonstrated that protein-bound CML and MG-H1 levels are higher in rupture-prone atherosclerotic plaques than in stable plaques. In addition, higher CML, CEL and MG-H1 levels were associated with higher levels of inflammatory plaque markers (MCP-1, IL-8 and MMP-9). However, plaque levels of CML, CEL and MG-H1 did not differ between individuals with and without T2DM. We also found that glyoxalase 1 (GLO1) mRNA was significantly decreased in ruptured plaques using an intra-plaque comparison, with a similar trend for protein and enzymatic activity levels.
- **Chapter 8.** We found that overexpression of GLO1 did not reduce AGEs nor atherosclerosis in ApoE deficient mice with and without streptozotocin (STZ) induced diabetes.

Discussion

The glyoxalase pathway in diabetes

The glyoxalase system, consisting of the enzymes GLO1 and glyoxalase 2, converts the major AGE precursor MGO, as well as glyoxal (GO), into D-lactate in the presence of reduced glutathione.¹⁰ Experimental studies have identified the glyoxalase system as an essential mechanism to protect cells from accumulation of MGO and AGEs in diabetes as well as in various other age-related disorders in which high metabolic activity takes place.⁹ However, expression and activity of the glyoxalase system in

individuals with and without T2DM had not been studied previously in large cohort studies.

Since no marker of the glyoxalase pathway suitable for use in large cohort studies existed (**Chapter 3**), we used UPLC-MSMS for the development of a sensitive and specific method to measure D-lactate, the end product of the glyoxalase pathway. With this technique we found that individuals with T2DM displayed higher D-lactate levels (**Chapter 3 and 5**). This finding was in line with results obtained using a previously described enzymatic assay.¹¹ These findings suggest that in T2DM increased levels of the AGE precursors, MGO and GO, are being detoxified.

Although we found an association between higher glucose and HbA1c levels and higher plasma D-lactate levels (**Chapter 5**), we cannot prove that D-lactate is a marker of MGO detoxification alone. D-lactate is also produced in the large intestine by fermenting gut-bacteria, which may contribute to circulating D-lactate levels.¹⁰ Indeed, we observed higher D-lactate levels in individuals with inflammatory bowel disease (**Chapter 3**). In addition, D-lactate may be present in some (fermented) foods or drinks, which may also end up in the circulation. Thus, a change in gut-microbiota or diet may have confounded the associations between D-lactate and either prevalent T2DM or CVD. However, we recently directly measured MGO levels in the CODAM study, and we found increased MGO levels in individuals with T2DM, suggesting that the increased levels of D-lactate in T2DM reflect increased detoxification of MGO.

We also included mRNA expression of GLO1, measured in circulating white blood cells, as an additional marker of the glyoxalase system. We found in the CODAM study that circulating GLO1 mRNA levels were higher in T2DM (**Chapter 5**). Whether this was due to changes in the numbers of circulating cell populations in individuals with T2DM is unknown, but this can be addressed in the future by adjusting for mRNA levels of markers such as CD45 (white blood cells) CD4 (T-cells), CD20 (B-cells), CD14 (monocytes) and CD66b (granulocytes). The increased GLO1 mRNA expression in the circulating cells may reflect a natural defense mechanism against increased exposure to MGO in the tissues although we do not know whether the increased GLO1 expression in the circulation translates to an increased enzymatic activity. Interestingly, higher GLO1 activity in monocytes and granulocytes of individuals with diabetic complications has been reported previously.¹² In contrast, no differences in red blood cell GLO1 activity between individuals with and without diabetes have been found.¹³ In a small study of individuals with type 1 diabetes (T1DM), increased GLO1 activity was found in a small subset of patients with retinopathy. In conclusion, detoxification of MGO is increased in individuals with T2DM, as reflected by increased levels of D-lactate and this may be partly explained by increased expression and activity of the glyoxalase system.

Surprisingly, experimental studies report decreased GLO1 expression in diabetic rodents.^{14,15} In line with these findings, we also found decreased GLO1 mRNA in the aortic arches of diabetic ApoE deficient mice (**Chapter 8**). Experimental diabetes as induced by STZ injections differs considerably from T2DM in humans, since it more

likely reflects T1DM as it is a model of insulin deficiency. Therefore, these preclinical studies should be interpreted with caution. The higher GLO1 expression levels in humans with T2DM may be due to frequent use of medications (such as candesartan, metformin and insulin), which have been linked to restoring GLO1 function.¹⁵⁻¹⁷ Although further adjustment for these medications did not change the associations between prevalent T2DM and GLO1 mRNA levels, we may have underestimated the effect of medication due to residual confounding. Furthermore, the decreased GLO1 expression in the animal studies was measured in tissues sensitive to diabetic complications, while human studies performed measurements in circulating cells. It might be that the increased GLO1 expression in circulating cells of individuals with T2DM is a compensatory mechanism against decreased tissue activity of GLO1 in diabetes.

The glyoxalase pathway in CVD

The protective role of GLO1 in preventing diabetic microvascular complications has been well established in experimental studies.¹⁸⁻²⁰ Whether GLO1 played a protective role in CVD as well had not yet been investigated.

We found no associations between GLO1 mRNA and cIMT, ABI or prevalent CVD, with similar findings for plasma and urinary D-lactate (**Chapter 5**). However, this does not rule out the possibility that GLO1 expression within the atherosclerotic plaque is associated with the phenotype of the atherosclerotic plaque itself. Therefore, we decided to further investigate the role of GLO1 in CVD by studying GLO1 mRNA expression in ruptured human atherosclerotic plaques (**Chapter 7**). Indeed, we found in ruptured plaque segments lower GLO1 mRNA levels, and GLO1 protein and activity levels showed similar trends. As we found *in vitro* that TNF and hypoxia decreased activity of GLO1 and increased AGE and MGO levels, we postulate that these metabolic triggers are also responsible for the decreased GLO1 expression within the plaque. The mechanisms regulating GLO1 activity in the plaque are likely to be complex, encompassing both regulation of gene expression,¹⁷ as well as post-translational modifications of the enzyme by agents such as nitric oxide and oxidized glutathione.²¹

Next, we investigated *in vitro* and *in vivo* potential causal contributions of the decreased GLO1 expression to development of plaque rupture. When we knocked down GLO1 by 40% in U937 monocytes, i.e. a similar decrease as observed with TNF incubation, this did not increase MGO levels. Therefore, the decreased GLO1 activity alone is insufficient to explain the increased AGE and MGO levels after exposure to hypoxia or TNF incubation. However, GLO1 knockdown did increase cytotoxicity of MGO in U937 cells. Therefore, we concluded that at least *in vitro*, the major role of GLO1 is to reduce cell death when MGO levels increase. We further investigated this postulate *in vivo* by crossing GLO1 overexpressing mice with ApoE deficient mice. We hypothesized that through prevention of cytotoxicity and through improved

endothelial function,¹⁸ GLO1 overexpressing mice would display smaller atherosclerotic lesions, with a stable phenotype. However, we rejected this hypothesis since we did not find any difference in atherosclerotic lesion size and phenotype, inflammation, or endothelial function between mice with and without GLO1 overexpression. In fact, we did not find any change in MGO or AGE levels as a result of GLO1 overexpression. This finding was in line with our GLO1 knockdown experiment *in vitro*, and confirms that MGO and AGE accumulation are largely independent of GLO1 in the setting of atherosclerosis. This is somewhat surprising since it has been consistently found that GLO1 overexpression is protective of microvascular complications in diabetic rodents,^{18-20,22,23} while GLO1 knockdown induced microvascular complications even in non-diabetic mice.^{14,20} Several reasons are suggested below as to why GLO1 overexpression affects micro- and macrovascular disease in a disparate manner:

- Firstly, we do not know whether ApoE deficient mice form as much MGO in their plaques as humans do. Therefore, it would be informative to investigate as to whether our huGLO1^{+/-} ApoE deficient mouse is protected against plaque growth and necrotic core formation when challenged with high MGO levels in the drinking water, as this procedure has been shown to increase atherosclerosis.²⁴ Based on our *in vitro* studies, we would predict the GLO1 overexpression will be protective in this setting.
- Secondly, as the ApoE deficient mouse model is characterized by a profound dyslipidemia, and lipid oxidation has been shown to be an important source of AGEs,²⁵ independently of glucose levels, it is likely that GLO1 overexpression may not be protective against AGE-formation in hyperlipidaemic conditions such as in ApoE deficient mice. GLO1 may not come into sufficient contact with the AGE precursors derived from lipid oxidation. This is likely, as GLO1 is present in the cytosol, where glycolysis takes place, but is not present in the cellular membranes and lipid particles where lipid oxidation largely takes place.²⁶ This hypothesis is testable by investigating whether GLO1 overexpression is protective against atherosclerosis in the absence of dyslipidemia, using a plaque regression study as recently performed albeit addressing a different issue.²⁷
- Thirdly, although we measured increased GLO1 activity in the aorta of the transgenic mice, the *in vivo* activity of GLO1 may be hampered by low availability of reduced glutathione, a possibility since atherosclerosis is generally characterized by oxidative stress.²⁸ Conversely, oxidized glutathione has been demonstrated to be a strong inhibitor of GLO1 activity.²⁹ In addition, GLO1 activity may be further reduced in the plaque by nitric oxide,³⁰ which is amply produced by macrophages. To investigate whether this is the case, it would be informative to treat the huGLO1^{+/-} ApoE deficient mouse with the major glutathione precursor N-acetylcysteine, and to investigate whether GLO1 overexpression would amplify the protective effect of N-acetylcysteine on atherosclerosis.³¹

Are AGE levels higher in individuals with type 2 diabetes?

The associations between high glucose levels and higher plasma AGE levels has been clearly shown in T1DM. Indeed, higher plasma AGE levels were found in T1DM,³² while glucose-lowering treatment was associated with lower AGE levels in the skin of individuals with T1DM.³³ In contrast, human studies on AGE levels in T2DM have reported mixed findings. Therefore, we addressed this issue in a large cohort study, measuring several AGEs with state of the art technology.

Although we found increased D-lactate levels in individuals with T2DM (**Chapter 4**), suggesting increased detoxification of MGO (and GO) levels, we found that protein-bound plasma levels of the specific AGEs CML, CEL and pentosidine were not increased in individuals with either impaired glucose metabolism or T2DM after adjustment for potential confounders (**Chapter 5**). We also found no consistent associations between fasting plasma glucose, HbA1c and any of the AGEs we measured. The lack of higher plasma AGEs in individuals with T2DM is in contrast to previous studies in T1DM and was considered a somewhat puzzling finding. Some of the most likely explanations as to why we did not find increased plasma levels of AGEs in T2DM are listed below:

- Compounds such as metformin, candesartan and simvastatin have AGE lowering properties,^{6,15,16,34} and are prescribed often to individuals with T2DM. Indeed, we found in the EPIC study that glucose-lowering treatment was associated with lower plasma AGE levels (particularly CML and pentosidine), and we found similar trends with blood-pressure lowering and lipid-lowering treatments (**Chapter 6**).
- Gaens et al. showed that plasma CML levels are lower in obese than in lean individuals, due to the fact that the adipose tissue takes up CML through a RAGE-dependent mechanism.³⁵ As obesity is strongly associated with the development of T2DM, but not T1DM, this phenomenon may explain why AGE levels were not elevated in T2DM, in contrast to T1DM. However, after adjusting for BMI no association between T2DM and plasma AGE levels was identified. However, since BMI and waist do not fully characterize adiposity, we may still have underestimated associations between T2DM and plasma AGEs.
- The recent publication of a popular book called “The AGE-less way” by Prof. Dr. Helen Vlassara has sparked a renewed interest in dietary AGEs. If the diet consumed by individuals with T2DM contained fewer AGEs (due to lifestyle interventions), while the endogenous formation of AGEs was increased, the net effect may be that we found no difference in AGE levels between individuals with and without T2DM. Whether this is a likely explanation of our findings remains to be determined, as it is far from clear as to what proportion of circulating AGEs is derived from dietary sources.
- The most likely explanation for the absence of increased AGE levels in T2DM is that AGE formation is not only dependent on glucose concentrations in T2DM but also derived from lipids,³⁶ or high metabolic activity at sites of inflammation. The latter may take place to a similar extent in individuals with and without diabetes, and may

override any potential differences in AGE levels attributable to hyperglycaemia. This concept is supported by our AGE measurements in atherosclerotic plaques (**Chapter 7**). Although we found no associations between plasma glucose or the presence of T2DM and plaque AGE levels, plaque AGEs were strongly associated with inflammatory markers and areas of hypoxia within the plaque. We also showed *in vitro* that inflammation and hypoxia contributed more to AGE accumulation than high glucose per se in U937 monocytes (**Chapter 7**), and that incubation of mouse macrophages with oxidized LDL moderately increased AGE-precursors (**Chapter 8**). These findings have some interesting implications for other diseases characterised by enhanced inflammation and/or metabolic activity, such as rheumatoid arthritis and many cancers. In addition, diseases hallmarked by hypoxia, such as chronic obstructive pulmonary disease and sleep apnea may also display increased AGE levels. Future studies should address whether AGE measurements could be used as markers of metabolic activity and disease progression in these other disorders.

Since we measured specific AGE moieties, we may have missed some AGEs that are really increased in T2DM. The sensitivity of certain tissues for AGE accumulation by high glucose levels may also differ. For example, we found in U937 monocytes no increase in AGEs after incubation with high glucose, while others have described this phenomenon in endothelial cells.³⁷ Whether AGE accumulation in circulating cells, microvascular structures such as the retina or glomeruli, or the skin is increased in T2DM remains to be determined in future studies. In this regard, skin autofluorescence, as a marker for AGE accumulation in the skin, has been repeatedly shown to be increased in T2DM.^{38,39} However, as not all fluorescent protein-modifications are AGEs, these results should be interpreted with due caution.

Associations between plasma AGEs and CVD

Experimental studies have supplied a framework as to how accumulation of AGEs may contribute to rupture of the atherosclerotic plaque (Figure 9.1). Indeed, animal studies have confirmed that AGE-inhibiting compounds reduce atherosclerosis in diabetic mice.^{40,41} However, human data on AGE accumulation and risk of CVD in individuals with and without diabetes are rather scarce.

Figure 9.1 Simplified schema as to how MGO and AGEs may link plaque inflammation to development of plaque rupture.

In this thesis, we found no associations between any of the measured plasma AGEs and prevalent CVD in the CODAM and Hoorn study (**Chapter 4**). However, we observed that protein-bound plasma levels of the AGEs CML and CEL were associated with incident CVD in the EPIC study (**Chapter 6**). In addition, we also found that plaque levels of certain AGEs, CML and MG-H1 were higher in plaques with a rupture-prone phenotype (**Chapter 7**). Furthermore, higher plaque AGE levels (CML, CEL and MG-H1) were associated with higher levels of inflammatory markers in the plaque (IL-8, MCP1 and MMP9).

Together, these findings indicate that in rupture-prone plaques more AGEs accumulate than in stable plaques, and that this process can (partly) be captured by measuring plasma AGEs. However, several confounding factors appear to weaken the association between plasma AGEs and incident CVD. In addition, several reasons need to be considered as to why no association with prevalent CVD was found. Firstly, AGE levels may not remain elevated after the development of a cardiovascular event, as several interventions to treat CVD have been shown to also reduce AGE levels.⁶ Another explanation comes from suggestions by Benzon et al.¹ These authors postulated that the macrophage content of the plaque is most subjected to regression. Therefore, markers reflecting plaque macrophages may display weaker associations with prior CVD than with incident CVD. This proposition actually is

consistent with our observation that the macrophage is the principal cell in which AGEs accumulate within the plaque. In order to develop AGE measurements into novel risk markers it is actually encouraging that they are not associated with prevalent CVD. Most risk predicting engines (such as the Framingham risk score) already include prevalent CVD as a strong predictor of incident CVD.⁴² Thus, AGE measurements may improve risk prediction since they do not reflect any process already included in the commonly used risk equations.

Although plasma AGEs in the free fraction are thought to better reflect tissue accumulation of AGEs than protein-bound plasma AGEs, associations between free plasma AGEs and prevalent CVD and T2DM were similar to that seen with protein-bound AGEs (**Chapter 4**). However, it remains to be determined as to whether this is also the case for incident CVD. Moreover, as free AGEs are largely cleared from the circulation by the kidneys,⁴³ it should also be investigated whether urinary levels of free AGEs are a better reflection of tissue AGE accumulation than plasma measurements. This is of interest, as free urinary AGEs would be attractive biomarkers due to easy accessibility of urine samples.

When comparing the results from the carotid plaques (**Chapter 7**) with the results from plasma AGE measurements, it is important to realize that although the protein-bound AGE moieties measured in plasma and plaques are identical, the proteins on which these modifications reside may be different. Specifically, the major plasma proteins are albumin, globulins and fibrinogen, whereas the major proteins in the plaque are derived from connective tissues, lipoproteins and cellular proteins. Therefore, AGE modification of albumin, which has a half-life of around twenty days, may reflect a different grade of AGE modification in comparison to proteins within the plaque. In this regard, measurement of AGEs in circulating cells may yield much stronger associations with CVD, as the AGE modifications on circulating cells are also likely to reside on cellular structures. In addition, circulating cells may be under similar metabolic stress (by low-grade inflammation, high glucose and lipid levels), as the atherosclerotic plaque in diabetes and/or obesity although these cells are unlikely to be challenged with the same harsh metabolic conditions found in the atherosclerotic plaque, such as prolonged hypoxia and high levels of oxidized lipoproteins. Therefore, the value of AGE levels in circulating cells as biomarkers for CVD remains to be determined. Since erythrocytes, neutrophils, monocytes and lymphocytes all have different half lives in the circulation and vastly different functions, measuring AGEs in these cellular subpopulations may yield different results. As these cellular fractions are difficult to separate, a promising initial step may be to compare associations between AGE levels measured in plasma, red blood cells and buffy coat and CVD.

Valorization

Future directions and applications of the glycation pathways to improve clinical management of T2DM and CVD

Using the glycation pathways to better predict CVD in individuals with and without diabetes

In an ideal situation we would be able to identify individuals at high risk of CVD, and treat them appropriately with risk-modifying medication and life-style changes to reduce their risk before an actual CVD-event takes place. Several risk-markers identify individuals at higher risk of CVD, such as older age, male sex, high blood-pressure, smoking and an unfavorable lipid profile (high LDL-cholesterol, low HDL-cholesterol).⁴⁴ In addition, diabetes itself is a strong risk-factor for CVD. Despite the identification of these risk markers and development of integrated risk-scores to calculate an estimated individual risk of CVD with these variables, it is still very difficult to precisely estimate which individuals will develop CVD and which individuals will remain event free, especially in individuals with diabetes.⁴⁵ As a result, many patients receive insufficient treatment, while others receive treatment from which they do not benefit. Therefore, it is important to study the underlying mechanisms of atherosclerosis, as this may help to identify new risk markers. When these risk markers reflect the underlying disease of a patient these risk markers are called biomarkers. Biomarkers, as measured in blood or urine of a patient, may improve the discrimination between patients with either a high or a low risk of cardiovascular events, which can subsequently be used to improve their treatment. This thesis provides a proof of principle that AGEs measured in the circulation may serve as biomarkers of increased risk of incident CVD. However, the plasma measurements we reported in this thesis displayed some limitations for use in clinical practice, such as associated confounding factors, including obesity. The possibility to measure plasma levels of AGE precursors, protein-bound and free AGEs in circulating cells and free AGEs in urine are all promising and essential steps to translate the findings summarized in this thesis into validated biomarkers which can actually improve risk prediction of CVD. This will be a major focus of future research. Future studies should also investigate the extent to which AGE measurements may serve as biomarkers for risk of microvascular complications of diabetes (retinopathy, neuropathy and nephropathy) in individuals with T2DM. Furthermore, as we identified inflammation and hypoxia as additional sources of AGE accumulation, AGE measurements may also be useful biomarkers in other diseases as well, such as rheumatoid arthritis and chronic obstructive pulmonary disease. In addition to identifying individuals at increased risk of CVD, AGE measurements may identify individuals that would particularly benefit from AGE-

lowering treatments. Such an approach may not only improve the identification of high-risk patients, but also the selection of optimal treatment strategies.

The glycation pathways as potential targets to treat CVD

As we show in this thesis that higher levels of specific AGEs are associated with a higher risk of CVD, it may be worth considering whether reducing AGE levels decrease CVD-risk. Several compounds have been identified that lower AGE levels and/or inhibit AGE formation. The best characterized inhibitor of AGE formation is aminoguanidine.⁴⁶ Administration of this compound has been shown to reduce plaque formation in diabetic ApoE^{-/-} mice.⁴⁰ In addition to AGE- and MGO-lowering properties, aminoguanidine has been described as an antioxidant⁴⁷ and inhibitor of nitric oxide formation.⁴⁸ Likewise, pyridoxamine (a vitamin B6 vitamer) is considered an AGE-inhibitor, with anti-oxidant and anti-inflammatory properties,⁴⁹ and has been shown to reduce atherosclerosis in diabetic ApoE^{-/-} mice.⁴¹ Alagebrium is a compound that has been reported to break pre-existing AGE-crosslinks and has also been shown to lower MGO levels directly.⁵⁰ Indeed, alagebrium has been shown to reduce atherosclerosis in diabetic ApoE^{-/-} mice, although it is unclear if its beneficial effect is attributable to its AGE- or MGO-lowering properties.⁴⁰

Whether these compounds also hold promise as treatment for CVD in humans has still not been determined.⁵¹ Unfortunately, aminoguanidine, when administered to people seemed to induce glomerulonephritis,⁵² and has therefore been deemed unsafe as a drug for humans. Therefore, there still is a need to develop and test new AGE inhibitors. Interestingly, established drugs to treat CVD, such as simvastatin, have also been shown to reduce the accumulation of AGEs in human carotid plaques.⁶ Similarly, several glucose-lowering¹⁶ and antihypertensive compounds³⁴ have also been shown to have AGE-lowering properties. Elucidating whether beneficial effects of these drugs are partly attributable to AGE-lowering is clearly relevant, as most of these drugs been shown to have beneficial effects beyond their glucose-,⁵³ lipid-,⁵⁴ and blood pressure-lowering.⁵⁵ Indeed, these characteristics could perhaps be further exploited by increasing their capacity to decrease AGE levels.

The reduction of dietary intake of AGEs may be an alternative strategy to reduce the risk of CVD. Especially processed foods as well as foods prepared by dry heat are thought to contain high levels of AGEs. Whether a diet low in AGEs or AGE-precursors leads to a reduction in AGE levels in the body is a topic that is currently hotly debated. Experimental studies have shown that the uptake of dietary AGEs is around 10-30%,⁵⁶ and that kidneys rapidly excrete excess free AGE fractions. Therefore, a diet low in AGEs may be particularly useful for patients with kidney failure. In animal studies, a diet low in AGEs reduces insulin resistance and atherosclerosis as well as diabetic neuropathy and nephropathy.⁵⁷ However, randomized clinical trials investigating beneficial effects of dietary regimens low in AGEs are rare, and contain only small

numbers of participants.⁵⁸ Therefore, larger clinical studies on this topic are urgently needed.

Based on findings reported in this thesis, boosting the activity of the glyoxalase pathway does not appear to be an attractive approach to treat CVD. As outlined earlier, we could not show any beneficial effect of GLO1 overexpression on plaque burden or phenotype in ApoE deficient mice. However, before we dismiss the glyoxalase pathway as a promising target in CVD, additional animal studies are needed to investigate the role of GLO1 in plaque regression and specifically to assess mice that are challenged to high MGO levels. An additional interesting direction for future research would be to develop compounds that remove inactivating posttranslational modifications from the GLO1 enzyme. Nevertheless, GLO1 remains an attractive target for diabetic microvascular complications.

In conclusion, the glycation pathways may be promising targets improve both prediction and treatment of CVD, and are therefore deserving of further investigation.

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Samenvatting

Samenvatting

Achtergrond informatie

Wereldwijd hebben maar liefst 382 miljoen mensen diabetes, met een verwachte toename tot 600 miljoen in 2035. Diabetes wordt gekenmerkt door verhoogde glucose (bloedsuiker) concentraties. Deze verhoogde glucose waarden zijn het gevolg van ofwel gebrekkige insuline productie (type 1 diabetes), of verminderde werkzaamheid van insuline (type 2 diabetes). De meeste mensen met diabetes hebben type 2 diabetes, dat meestal ouderen of mensen met overgewicht treft, terwijl 10% van de mensen met diabetes aan type 1 diabetes lijdt. Echter, ook type 2 diabetes komt op steeds jongere leeftijd voor. De sterkste toename van type 2 diabetes wordt in Afrika, Azië en Zuid Amerika waargenomen. Hoewel vooral type 2 diabetes in het begin weinig symptomen kan hebben, is diabetes bepaald geen onschuldige ziekte. Diabetes kan leiden tot een aantal ernstige complicaties, die helaas vaak voor komen. Diabetische nierschade (nefropathie) is een van de meest voorkomende nierziekten. Diabetische oogschade (retinopathie) is in de westerse wereld een van de meest voorkomende oorzaken van blindheid. Daarnaast komen diabetische zenuwschade (neuropathie) en voetzweren ook veel voor; diabetes is een belangrijke oorzaak voor amputaties van de onderste ledematen. Deze aandoeningen hebben een ernstig beperkend effect op de levensduur en levenskwaliteit van mensen met diabetes. De belangrijkste doodsoorzaak bij mensen met diabetes zijn hart- en vaatziekten, zoals een hartinfarct of beroerte. Door toename van risicofactoren die de kans op zowel diabetes en hart- en vaatziekten verhogen, zoals overgewicht, roken, hoge bloeddruk en ouderdom, is de verwachting dat sterfte en maatschappelijke belasting door deze complicaties alleen maar groter wordt.

De belangrijkste oorzaak voor het ontwikkelen van hart- en vaatziekten is het openscheuren van een aderverkalking (de technische term is atherosclerotische plaque). Dit proces begint met ophoping van cholesterol op bepaalde plekken in de slagaders, dat grote hoeveelheden ontstekingscellen aantrekt (vooral een celtype genaamd macrofaag). Deze cellen nemen het cholesterol op en proberen het te verteren. Omdat het cholesterol sneller ophoopt dan de macrofagen het kunnen opruimen, hoopt zich steeds meer cholesterol op in de macrofagen. De macrofagen zullen uiteindelijk zelfs sterven. Omdat op hun beurt ook de macrofagen onvoldoende worden opgeruimd, wordt er een necrotische kern gevormd in de aderverkalking. Zoals bij de meeste processen waarbij weefselsterfte plaats vindt, treedt hierbij een beschermingsmechanisme in werking waarbij het ontstekingsproces wordt ingekapseld door een laag bindweefsel en gladde spiercellen, ook wel fibreuze kap genaamd. Een klassieke misvatting is dat het steeds verder aangroeien van de plaque er uiteindelijk voor zorgt dat de slagader helemaal afgesloten wordt, waardoor de bloedtoevoer afgesloten wordt en door zuurstofgebrek een hartinfarct of beroerte ontstaat. Echter, omdat aderverkalking een traag proces is, dijt de diameter van een

bloedvat vaak met de groeiende plaque mee uit, en tegelijkertijd ontstaan voldoende natuurlijke omleidingen waarlangs het bloed kan blijven stromen. De fibreuze kap blijkt helaas niet altijd sterk genoeg om de onderliggende necrotische kern ingekapseld te houden. Als deze fibreuze kap scheurt, komt de necrotische kern in contact met het bloed, en dit leidt tot onmiddellijke activatie van de bloedstolling, waardoor het bloedvat direct afgesloten wordt en acuut zuurstof gebrek optreedt. We nemen aan dat hoe groter de necrotische kern is, en hoe dunner de fibreuze kap, des te groter het risico op het scheuren van de aderverkalking is. We weten echter maar weinig over de directe oorzaken van scheuring, en we weten ook niet waarom sommige aderverkalkingen een dikke fibreuze kap hebben, terwijl andere aderverkalkingen vooral een grote necrotische kern hebben.

Het is bekend dat in aderverkalkingen een sterk verhoogde stofwisseling plaats vindt, en veel glucose verbruikt wordt om het ontstekingsproces van voldoende brandstof te voorzien. Het is bekend dat in condities van sterk verhoogde stofwisseling versuikerde eiwitten (afgekort AGEs) ophopen. Omdat aan AGEs verschillende schadelijke effecten worden toegeschreven, zou ophoping van AGEs kunnen bijdragen aan het scheuren van aderverkalkingen waarin een hoge mate van ontsteking optreedt.

AGEs behoren tot een grote verzameling van chemische verbindingen die tot stand komen wanneer suikers met eiwitten reageren. Dit proces is wellicht het meest bekend in voedselbereiding. Voorbeelden zijn de Merengue en de bruine korst die ontstaat bij het bakken van brood of het grillen van vlees. Deze zogeheten bruiningsreactie door verhitting van eiwitten en suikers werd ontdekt door Louis Camille Maillard in 1912. Omdat ons lichaam “maar” 37 graden is, verloopt deze reactie in ons lichaam zeer traag. In de jaren tachtig van de vorige eeuw werd ontdekt dat deze reactie plaats vindt in weefsels die langzaam vernieuwd worden, zoals het bindweefsel in de huid en bloedvaten, en bijdraagt aan veroudering van deze weefsels. Uit verder onderzoek bleek echter dat tussenproducten van de suiker-afbraak vele malen sneller reageren met eiwitten tot AGEs dan glucose zelf. Van deze AGE voorlopers is methylglyoxaal de belangrijkste en meest reactieve voorloper. Het ophopen van AGEs wordt in verband gebracht met het ontwikkelen van verschillende verouderingsziekten (van dementie tot huidveroudering), en met de complicaties van diabetes in het bijzonder. Dierproefstudies lieten ook zien dat verschillende stoffen die AGE vorming tegengaan, de vorming van aderverkalking remmen. In de plaque zouden AGEs op verschillende wijzen bij kunnen dragen aan de ontwikkeling van plaque scheuring. Er bestaat bijvoorbeeld een receptor voor AGEs (RAGE). Als deze receptor geactiveerd wordt leidt dit tot een activatie van het ontstekingssysteem van de cel. Daarmee zou de ontstekingsreactie in de plaque verder aangejaagd kunnen worden. Inderdaad, muizen die deze receptor door genetische modificatie niet hebben zijn in zeer sterke mate beschermd tegen aderverkalking en complicaties van diabetes. Ten tweede is methylglyoxaal in hoge concentraties giftig, en zou dus bij kunnen dragen aan toegenomen weefselversterfte in de aderverkalking en daarmee aan versnelde aangroei van de necrotische kern. Gelukkig kan methylglyoxaal worden

afgebroken door de cel. Belangrijk in dit verband is het glyoxalase systeem, dat overmatige AGE vorming tegengaat door methylglyoxaal af te breken tot rechtsdraaiend melkzuur. Deze chemische reactie verloopt in twee stappen, waarbij de eerste stap, die voltrokken wordt door het enzym glyoxalase 1 (GLO1), de snelheidsbepalende stap is. Het belang van dit systeem wordt onderstreept door het vroege ontstaan van het glyoxalase systeem in het evolutionaire proces, en daardoor aangetroffen wordt in bacteriën, planten, schimmels en dieren. Echter, het glyoxalase systeem bevat enkele zwakheden waardoor het niet altijd goed in staat lijkt te zijn de vorming van methylglyoxaal tegen te gaan. Ten eerste is voor de GLO1 reactie glutathion nodig. Omdat deze stof bij langdurig verhoogde stofwisseling op kan raken, kan de GLO1 reactie hierdoor onvoldoende plaats vinden. Ten tweede is de GLO1 activiteit bevattelijk voor inactivatie door allerlei bijproducten van verhoogde stofwisseling. Ten derde wordt de productie van GLO1 gereguleerd door tal van signalen die de cel ontvangt, van ontstekingsignalen tot insuline. Deze factoren samen kunnen bijdragen aan onvoldoende bescherming tegen AGE vorming in de cel. AGEs afgeleid uit de reactie van methylglyoxaal met aminozuren hoeven dus niet alleen een indicatie van hoge suikerconcentraties te zijn, maar kunnen ook ontstaan door het laten afweten van het GLO1 systeem door bijvoorbeeld ontsteking of bij zuurstof gebrek. Omdat zowel aderverkalking als diabetes gekenmerkt worden door ontsteking en (lokaal) verhoogde stofwisseling, is het aannemelijk dat het glyoxalase systeem zijn werkzaamheid verliest en daardoor AGE ophoping versneld plaats kan vinden in deze aandoeningen.

Belangrijkste bevindingen uit dit proefschrift

In dit proefschrift werd onderzocht of metingen van AGEs en het glyoxalase systeem, in bloed en in weefsels, samenhangen met het hebben van diabetes en ontwikkelen van hart- en vaatziekten.

In dit proefschrift hebben we gebruik gemaakt van grote bevolkingsonderzoeken om de verbanden tussen AGEs, diabetes en hart- en vaatziekten verder te onderzoeken. In dit proefschrift hebben we ook gebruik gemaakt van indirecte metingen van het hebben van aderverkalking, als een maat voor vroege manifestaties van hart- en vaatziekten. Een van die maten, de enkel-arm index, is veelgebruikt omdat deze eenvoudig te meten is en sterk met hart- en vaatziekte samenhangt. Kortgezegd wordt de bloeddruk in de arm gedeeld door de bloeddruk in de benen, als maat voor vernauwingen in de beenslagaders door aderverkalking. In **hoofdstuk 2** werd eerst onderzocht of de enkel-arm index even sterk samenhangt met sterfte door hart- en vaatziekten in mensen met diabetes als mensen zonder diabetes. Dit was een belangrijke vraag omdat er aanwijzingen bestonden dat de enkel-arm index minder betrouwbaar zou zijn in mensen met diabetes en we in een latere studie in dit proefschrift van de enkel-arm index gebruik maken in mensen met- en zonder diabetes. Uit **hoofdstuk 2** blijkt dat de enkel-arm index even sterk samenhangt met

algemene sterfte en sterfte door hart- en vaatziekten in mensen met diabetes als in mensen zonder diabetes.

Het volgende doel was om uit te zoeken of AGEs inderdaad wel verhoogd waren in mensen met type 2 diabetes. In **hoofdstuk 4** werd beschreven dat een aantal AGEs gemeten in plasma niet verhoogd blijken te zijn in mensen met type 2 diabetes. Dit resultaat was verassend, en in tegenstelling met studies uitgevoerd in patiënten met type 1 diabetes. Bovendien, werd ook geen enkel verband gevonden tussen de AGEs en eerder ontwikkelde hart- en vaatziekten.

Vervolgens werd onderzocht of het hebben van type 2 diabetes het glyoxalase systeem beïnvloedt. Daartoe werd eerst in **hoofdstuk 3** een zeer gevoelige techniek beschreven om lage concentraties van rechtsdraaiend melkzuur, het eindproduct van het glyoxalase systeem, betrouwbaar te kunnen meten in plasma en urine. Met massaspectrometrie bleek het goed mogelijk om links- en rechtsdraaiend melkzuur van elkaar te onderscheiden en betrouwbaar te meten. Bovendien bleken zowel linksdraaiend als rechtsdraaiend melkzuur verhoogd in plasma en urine van mensen met type 2 diabetes. In **hoofdstuk 5** werd onderzocht of het glyoxalase systeem samenhangt met het hebben van type 2 diabetes en met indicatoren van aderverkalking zoals de enkel-arm index. Als afspiegelingen van het glyoxalase systeem werd mRNA van GLO1 gemeten in het bloed, en rechtsdraaiend melkzuur gemeten in plasma en urine. Hoewel GLO1 mRNA en wederom rechtsdraaiend melkzuur verhoogd bleken in mensen met type 2 diabetes, bleek geen van deze metingen op consistente wijze samen te hangen met de verschillende maten van aderverkalking.

In **hoofdstuk 6**, in een studie uitgevoerd in mensen met type 2 diabetes bleek dat AGEs gemeten in plasma wel samen hangen met het ontwikkelen van toekomstige hart- en vaatziekten. Deze bevindingen werden verder bevestigd in **hoofdstuk 7**. Hierin vonden we ook een samenhang tussen AGE concentraties gemeten in plaques en verschillende eigenschappen van deze plaque. Twee belangrijke AGEs bleken inderdaad verhoogd te zijn in plaques met kenmerken van ruptuur. Er werd geen verdere verhoging van AGEs in de plaques afkomstig van mensen met type 2 diabetes aangetroffen. Aankleuringen van AGEs werden voornamelijk aangetroffen rondom de kern van de plaque en vooral in gebieden waar lage zuurstof spanning heerst, en die rijk zijn aan macrofagen. Bovendien werd een sterk verband gevonden tussen hogere waarden van AGEs en hogere waarden van ontstekingsmarkers gemeten in de plaque. Bovendien bleek dat mRNA van GLO1 verlaagd is in segmenten van plaques waar scheuring had plaats gevonden, vergeleken met segmenten die als stabiel waren beoordeeld. Een soortgelijk patroon werd ook aangetroffen voor GLO1 eiwit waarden en enzymatische activiteit. Uit experimenten uitgevoerd in het laboratorium met gekweekte macrofagen bleek dat ontsteking en zuurstofgebrek inderdaad belangrijke oorzaken voor de vorming van methylglyoxaal en AGEs kunnen zijn. Dit ging ook samen met verlaging van GLO1 activiteit. Vervolgens bleek methylglyoxaal in hoge concentraties in staat de macrofagen te doden. Echter, we vonden in celweek dat

toevoegen van hoge suikerconcentraties aan het kweekmedium niet leidt tot een verhoogde aanmaak van AGEs. Deze bevindingen kwamen overeen met de waarnemingen in de aderverkalkingen, en suggereren dat de hoge mate van ontsteking en zuurstofgebrek in de plaque bijdragen aan de vorming van methylglyoxaal, dat vervolgens tot AGE vorming en weefselversterf kan leiden. Bovendien is een belangrijke bescherming hiertegen, het enzym GLO1, afgenomen. Dit zou vervolgens tot een vicieuze cirkel kunnen leiden waarbij hoge mate van ontsteking tot een steeds verdere toename van methylglyoxaal en weefselsterfte leidt, met uiteindelijk scheuring van de aderverkalking en een beroerte als gevolg. In **hoofdstuk 8** werd onderzocht of daling van GLO1 in gescheurde aderverkalking inderdaad een oorzakelijke rol speelt in het ontstaan van aderverkalking zelf, en in het toenemen van kenmerken die duiden op een verhoogd risico op scheuring. Om deze vraag te beantwoorden, werden muizen die veel GLO1 produceren gekruist met muizen die aderverkalking ontwikkelen. We vonden echter geen enkel verschil in mate van aderverkalking tussen muizen met en zonder GLO1 overexpressie.

Conclusie

Dit proefschrift bevestigt dat eerdere bevindingen over de rol van AGEs in aderverkalking in proefdieren ook op lijken te gaan voor mensen, en wijst nieuwe mechanismen aan waarmee AGEs aan scheuring van de aderverkalking zouden kunnen bijdragen. Samengevat, de belangrijkste bevindingen van dit proefschrift zijn dat hogere AGE concentraties in het plasma samenhangen met een hoger risico op het ontwikkelen van hart- en vaatziekten en inderdaad ophopen in plaques met een hoog risico op scheuring. Hoewel het GLO1 systeem verlaagd blijkt te zijn in gescheurde aderverkalkingen, en ontsteking en zuurstof gebrek hier mogelijke oorzaken voor zijn, bleek een verhoogde concentratie van GLO1 niet te beschermen tegen aderverkalking in muizen. Hiermee wordt de GLO1 daling als directe oorzaak voor de AGE ophopingen in aderverkalking enigszins in twijfel getrokken. Toekomstige medicijnen om AGE vorming te remmen zullen zich dus waarschijnlijk direct op AGE vorming zelf moeten richten, in plaats van ondersteuning van het glyoxalase systeem. Zoals aangegeven bestaan er een aantal potentiële medicijnen die de vorming van methylglyoxaal en AGEs tegen kunnen gaan. Dit proefschrift onderstreept het potentiële belang van deze verbindingen om diabetes en hart- en vaatziekten te bestrijden.

Dit proefschrift laat ook zien dat AGE metingen in plasma mogelijk geschikt kunnen zijn om hart- en vaatziekten te voorspellen. Dit is vooral belangrijk voor mensen met diabetes, omdat nog altijd zeer lastig te voorspellen is wie het hoogste risico op het ontwikkelen van hart- en vaatziekten heeft. Door gebrekkige risicoschatting krijgen veel mensen met diabetes hierdoor niet genoeg, of juist te veel medicijnen om hun risico op hart- en vaatziekten te beperken. Het is belangrijk te vermelden dat we maar een kleine selectie van AGEs hebben gemeten in dit proefschrift. Mogelijk zijn er

andere AGEs die wel in type 2 diabetes verhoogd zijn en nog sterker met hart- en vaatziekten samenhangen. Bovendien, vele technische verfijningen van de huidige meetmethoden, en metingen in bloedcellen of urine in plaats van plasma, en het direct meten van methylglyoxaal zijn mogelijk om hier verbetering in aan te brengen, en dit zal dan ook de inzet zijn van toekomstig onderzoek.

Dankwoord

Dankwoord

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Curriculum Vitae

Curriculum Vitae

Nordin Hanssen was born on 28-3-1985 in Sittard, the Netherlands. He attended the Tevianum gymnasium in Sittard, and graduated in 2003. He studied medicine from 2003-2009 at Maastricht University. From 2009 till 2013 he worked as a PhD student at the department of internal medicine, Maastricht University, under supervision of Prof. dr. Casper Schalkwijk, Prof. dr. Coen Stehouwer and Dr. Kristiaan Wouters. During this time, he obtained in part-time a master's degree in epidemiology at Maastricht University, and graduated in 2011. In 2013-2014, he worked for 6 months as a visiting PhD student at the Baker IDI research institute for diabetes and cardiovascular disease in Melbourne, Australia, under supervision of Prof. dr. Mark Cooper, Prof. dr. Assam El-Osta and Dr. Andrew Murphy. From August 2014 onwards he started his residency in internal medicine in the Maastricht University Medical Centre (MUMC+) under supervision of Prof. dr. Coen Stehouwer and Prof. dr. Richard Koopmans.

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- DE Maessen, **NMJ Hanssen**, JJM Scheijen, CJH van der Kallen, MMJ van Greevenbroek, CDA Stehouwer, CG Schalkwijk. Post-glucose plasma α -dicarbonyl concentrations are increased in individuals with impaired glucose metabolism and type 2 diabetes: The CODAM study, *Diabetes Care*. 2015 *In press*
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Grants

- *European Foundation for the Study of Diabetes (EFSD) travelgrant*
Adverse effect of high glucose on circulating cells and inflammatory cytokines in C57Bl6 mice and bone marrow derived macrophages, does SET7 play a role?
- *International Atherosclerosis Society travelgrant*
Do methylglyoxal-induced epigenetic changes in bone marrow derived macrophages contribute to cardiovascular disease in diabetes?
- *De drie lichten stichting grant*
Do methylglyoxal-induced epigenetic changes in bone marrow derived macrophages contribute to cardiovascular disease in diabetes?

International oral presentations

- *American Diabetes Association, 2013 (Chicago)*: Levels of Advanced Glycation Endproducts in carotid atherosclerotic plaques of individuals with and without diabetes correlate with characteristics of rupture-prone lesions
- *IMARS, international symposium on the Maillard reaction, 2012 (Nancy)*: Levels of Advanced Glycation Endproducts in carotid atherosclerotic plaques correlate with characteristics of rupture-prone lesions
- *European Association for the Study of Diabetes, 2012 (Berlin)*: Plasma levels of advanced glycation endproducts are not independently associated with cardiovascular disease in individuals with or without type 2 diabetes: The Hoorn and CODAM Studies

Abbreviations

Abbreviations

ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
AGEs	Advanced glycation endproducts
ALEs	Advanced lipid oxidation products
ACE	Angiotensin converting enzyme
ARBs	Angiotensin receptor blockers
ABI	Ankle-brachial blood pressure index
BMI	Body mass index
CEL	Nε(carboxyethyl)lysine
CAT	Catalase
CI	Confidence interval
CODAM	Cohort study of Diabetes and Atherosclerosis Maastricht
CHF	Congestive heart failure
CHD	Coronary heart disease
cIMT	Carotid intima media thickness
CML	Nε(carboxymethyl)lysine
CVD	Cardiovascular disease
CEA	Carotid endarterectomy
CV	Coefficients of variation
CRP	C-reactive protein
DBP	Diastolic blood pressure
EPIC	European Prospective Investigation into Cancer and Nutrition
FACS	Flow cytometry
eGFR	Glomerular filtration rate
GMS	Glucose metabolism status
Gpx1	Glutathione peroxidase 1
HbA1c	Glycated hemoglobin
GO	Glyoxal
GLO	Glyoxalase
HR	Hazard ratios
HMOX1	Heme oxygenase 1
HDL	High density cholesterol
HPLC	High-performance liquid chromatography
IGM	Impaired glucose metabolism
IBD	Inflammatory bowel disease
IL-6	Interleukin 6
IL-8	Interleukin 8
IDF	International Diabetes Federation
IQR	Interquartile range
Keap1	Kelch-like ECH-associated protein 1

LDL	Low density cholesterol
MMP	Matrix metalloprotease
MG-H1	5-hydro-5-methylimidazolone
MGO	Methylglyoxal
MAP	Mean arterial pressure
MCP1	Monocyte chemotactic protein-1
NQO1	NAD(P)H dehydrogenase (quinone1)
NGM	Normal glucose metabolism
NRF2	Nuclear factor erythroid 2-related factor 2
NAC	<i>N</i> -acetylcysteine
OR	Odds ratio
PAD	Peripheral arterial disease
RAGE	Receptor for AGEs
RR	Relative risk
RIN	RNA integrity number
SD	Standard deviation
SEM	Standard error of the mean
SBP	Systolic blood pressure
SAA	Serum amyloid A
sE-selectin	Soluble E-selectin
sICAM-1	Soluble intercellular adhesion molecule 1
sVCAM-1	Soluble vascular adhesion molecule 1
TBI	Toe brachial index
TNF	Tumour necrosis factor
T2DM	Type 2 diabetes
UPLC-MSMS	Ultra performance liquid chromatography tandem mass spectrometry
vWF	Van Von Willebrand factor
WHR	Waist-hip ratio