Intervention strategies in the glycation pathway with methylglyoxal as the primary target

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Intervention strategies in the glycation pathway with methylglyoxal as the primary target

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Chapter 1

General introduction

General introduction

Chronic metabolic diseases like diabetes mellitus and cardiovascular disease (CVD) form one of the largest health burdens of the 21st century[1,2]. Overweight and obesity are central etiological factors in the development of these chronic metabolic diseases. In 2016 almost two billion people suffered from overweight[3]. Because of the increase in both life expectancy and prevalence of these chronic metabolic diseases, the number of years lived with diabetic and vascular complication has risen, and the overall disease burden keeps growing. Consequently, there is a continuous need for improved and preventive treatments[4,5].

A better understanding of the development from overweight and obesity towards diabetes and vascular complications can help to improve current conducts. The WHO states, *"Diabetes can be treated and its consequences avoided or delayed with diet, physical activity, medication and regular screening and treatment for complications"*. Non enzymatic glycation and the formation of AGEs is believed to be one of the main mechanisms in the development and consequences of chronic metabolic diseases. In this thesis, I studied the effects of weight loss, physical activity, and nutraceutical interventions on non-enzymatic glycation.

I. Pathophysiological background

Diabetes and cardiovascular disease

Diabetes is a chronic metabolic disease characterized by elevated plasma glucose levels resulting from defective insulin secretion or insulin signalling. In 2021, one out of ten adults has diabetes[6]. About 5-10% of individuals with diabetes have type 1 diabetes. Type 1 diabetes is an autoimmune disease targeting the insulin-producing cell of the pancreas[7]. This leads to an absolute insulin deficiency and becomes life threatening in a short period of time. The majority (~90%) of individuals with diabetes have type 2 diabetes. This form of the disease can develop over a long period. The risk of developing type 2 diabetes increases with age, obesity, and lack of physical activity[7]. Characterized by a deteriorating insulin production and insulin sensitivity of the body, type 2 diabetes can lead to life-threatening complications. The rising incidence of diabetes (and consequent complications) can be attributed largely (~70%) to the growing and ageing population. From an etiological standpoint, obesity and physical inactivity are primary risk factors in the development of type 2 diabetes mellitus[8,9]. Next to type 2 diabetes, the term 'prediabetes' serves as an additional risk classification based on blood glucose levels, but with less severe hyperglycaemia than individuals with type 2 diabetes. People

with prediabetes will often meet several criteria of the metabolic syndrome, and this classification can serve as a 'red flag' in the prevention of diabetes and vascular complications.

Among many possible diabetic complications, cardiovascular disease (CVD) is the most prevalent comorbidity[10]. Furthermore, CVD is the major cause of mortality in the western world, with stroke and heart attack as common manifestations. CVD is usually associated with accumulation of fat deposits in the arteries, increased risk of blood cloths and increased risk of arterial damage in major organs like the heart, brain, kidneys and eyes. Coronary heart disease, strokes and TIAs, peripheral arterial disease, and aortic disease are the four main categories of CVD.

Microvascular dysregulation

The microcirculation includes capillaries, arterioles and venules. The primary function of the microcirculation is the supply of oxygen, nutrients, metabolites and uptake of waste products in response to variations in metabolic demand. This response is primarily regulated via vasodilation and vasoconstriction, regulating the blood supply to the surrounding tissue. The microcirculation also regulates hydrostatic pressure, and is thus responsible for the peripheral resistance. Endothelial cells are critical in the pathophysiology of microvascular disease as they are the barrier between the circulation and organs. Endothelial cell function and microvascular function are very closely related. Examples of severe microvascular complications of diabetes include nephropathy, retinopathy, neuropathy, and peripheral vascular disease in the lower limbs and feet.

The loss of the aforementioned vasoregulatory mechanisms results in the development of microvascular dysfunction and can affect peripheral vascular resistance and thus blood pressure, predominantly by microvascular rarefaction (reduced capillary density) and altered flow patterns in the microcirculation[11,12]. Changes in muscle perfusion and non-uniform distribution of blood flow can also affect nutrient and hormone access to muscle tissue and metabolic function. Impaired capillary recruitment can cause an impairment in glucose uptake by the muscle, resulting in vascular insulin resistance[13,14].

The effect of insulin is in part determined by the ability to reach metabolic active tissue through changes in local blood flow distribution. The microvascular actions of insulin in fact promote its own delivery, and that of glucose, to skeletal muscle cells, thereby increasing insulin- mediated glucose disposal[15-18]. Insulin not only stimulates GLUT4 translocation to the cell surface, but it also increases NO synthesis in vascular endothelial cells, causing relaxation of precapillary terminal arterioles and redirecting blood flow from non-nutritive to nutritive microvessels[16,19]. In healthy individuals, the net physiological effect of insulin on the microvasculature is vasodilation. The increased synthesis of NO through the phosphatidylinositol-3-kinase (PI3K) pathway (with

vasodilation as a result)[20,21], outweighs the increase in endothelin synthesis through the extracellular signal-regulated kinase (ERK) pathway (with vasoconstriction as a result). In obese individuals, insulin-mediated muscle microvascular recruitment is impaired[22-24], adiponectin levels are reduced, and free fatty acids and TNF-α are increased, impairing endothelial cell signal transduction and dysregulating the balance in favour of the ERK pathway, resulting in a disturbed effect of insulin with more vasoconstriction in the microvasculature[25-30]. This process is called vascular insulin resistance[31]. In addition to direct effect of pro-inflammatory cytokines, FFAs, and elevated dicarbonyl and oxidative stress, also give rise to endothelial damage and impaired microvascular function (both impaired capillary recruitment and impaired microvascular vasodilation)[16] (Figure 1.1).

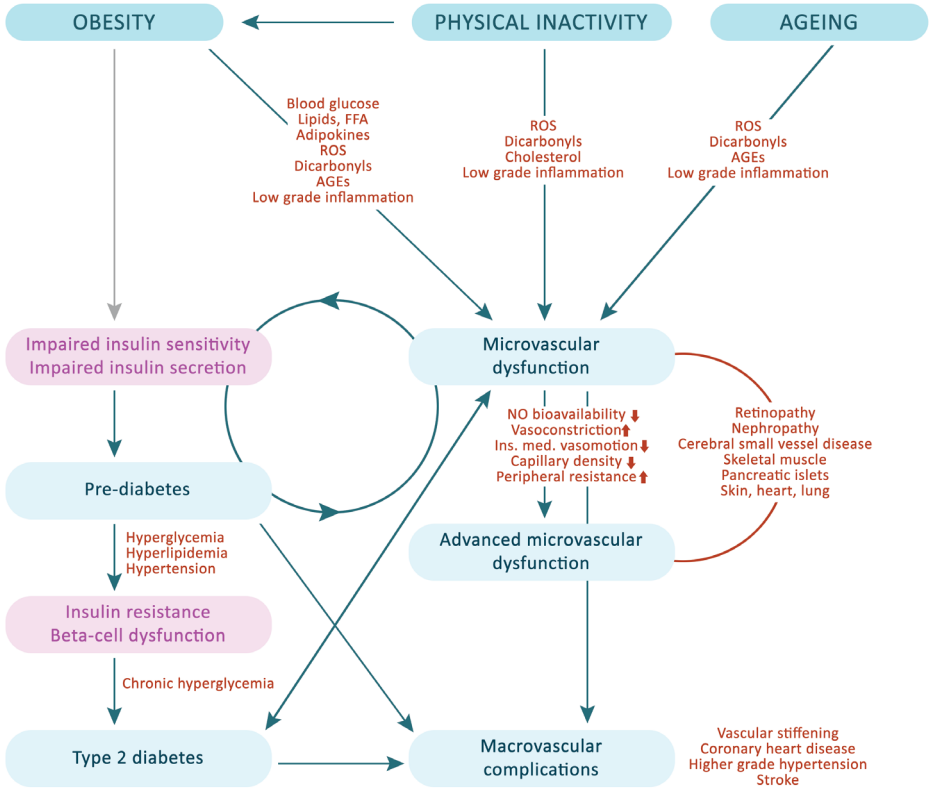

Figure 1.1 Overview of the risk factors and pathogenesis of diabetes and vascular disease, focused on obesity-associated impaired insulin and impaired glucose regulation, and consequent microvascular complications. Considering disease etiology, next to obesity, physical activity and ageing, other factors also play a role. Diet, alcohol consumption, smoking, genetic predisposition, early life exposure, and large artery stiffening can all contribute to (micro)vascular dysfunction[32]. Figure adapted from[32,33].

Microvascular and metabolic (glucose) regulation are interconnected. Metabolic dysregulation resulting from hyperglycemia and hyperlipidemia, causes microvascular dysfunction. Microvascular dysfunction impairs insulin-mediated glucose disposal and glucose-induced insulin secretion, which can ultimately cause insulin resistance and impaired insulin secretion, creating a vicious cycle[34] (Figure 1.1). Impaired microvascular endothelial function is associated with incident type 2 diabetes, and even impaired fasting plasma glucose[35].

The good news is that microvascular dysfunction in obesity is partly reversible by diet-induced weight loss[24,36], making it an interesting target in the prevention of the metabolic syndrome and type 2 diabetes. In this thesis, we focus on the contribution of the glycation pathway to microvascular dysfunction.

Adipocytes in obesity

White adipose tissue is capable of storing large amounts of lipids and is primarily known for its role as fat-storage and its large plasticity in this function. In fact, adipose tissue is a very active endocrine organ, producing cytokines (adipokines), hormones and growth factors[37,38]. A dysregulation of these signalling molecules links the adipose tissue to the microcirculatory damage, and insulin resistance.

When excessive amounts of calories are consumed and the subcutaneous adipose storage capacity becomes saturated, the visceral (abdominal) adipose tissue and other ectopic fat depots can show rapid expansion and consequently develop a metabolic disturbance associated with the development of insulin resistance in obese individuals[39,40]. This relation can be explained by the creation of a hypertrophic, pro-inflammatory environment in the adipose tissue. When the adipose tissue is unable to cope with the increased amount of fatty acids, a disproportional amount of hypertrophy and insufficient angiogenesis causes impaired adipogenesis, resulting in adipocyte hypertrophy and hypoxia and a dysregulation of adipokine levels[41]. Among others, free fatty acids (FFAs), leptin, resistin, angiotensin II, TNF-α, and plasminogen activator inhibitor-1 (PAI-1) are increased in the 'inflamed' adipose tissue, while adipose tissue in lean individuals tends to secrete anti-inflammatory adipokines like adiponectin[41-44].

Both systemically and at the source in the perivascular fat, this pro-inflammatory state links increased adiposity to early damage to the endothelium and microcirculation[45]. Furthermore and in the context of this thesis, the pro-inflammatory adipose environment is an important source of the endocrine production of dicarbonyls and AGEs.

II. The glycation pathway

Many mechanisms have been described explaining the higher risk of vascular complications in diabetes. Brownlee described a unified process proposing increased activity in four major pathways as a pathophysiological link between hyperglycemia and vascular damage (Figure 1.2)[46]. These are the polyol pathway, the protein kinase C (PKC) pathway, the hexosamine pathway and the glycation pathway. The dysregulation of these four pathways is a consequence of metabolites formed during glycolysis. Hyperglycemia induces an overproduction of superoxide by the mitochondrial electron-transport chain; this partially inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[47] and causes an increased flux in the four pathways. An increased flux of glucose in the glycolysis pathway also results in an increase in triose phosphates and causes elevated intracellular levels of the reactive dicarbonyl methylglyoxal (MGO). MGO is the main intracellular AGE precursor.
In this thesis, we focus on the effects of the glycation pathway in metabolic and vascular disease and on interventions to reduce methylglyoxal and AGEs.

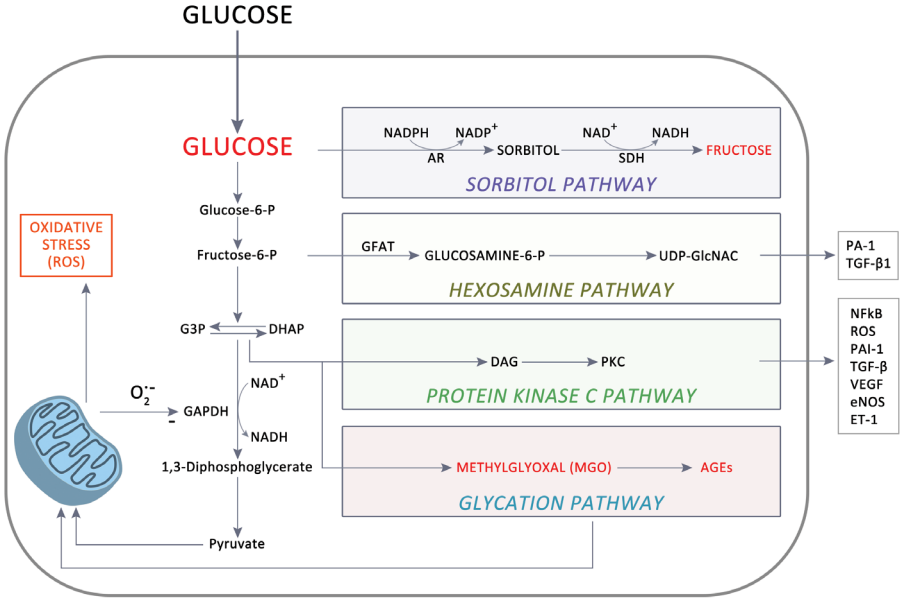

Figure 1.2 Overview of the four pathways driving hyperglycemic damage, adapted from[44]. The polyol (sorbitol) pathway, the hexosamine pathway, the protein kinase C (PKC) pathway, and the glycation pathway form a pathophysiological link between hyperglycemia and vascular damage. Hyperglycemia induces an overproduction of superoxide by the mitochondrial electron-transport chain; this partially inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[45] and causes an increased flux in the four pathways.

Advanced glycation endproducts (AGEs)

AGEs can be formed through several distinct metabolic pathways, most related to glucose metabolism and dicarbonyl compounds (Figure 1.3). The classic Maillard reaction, described by Louis Camille Maillard in 1912[48], is the slow, non-enzymatic reaction of glucose or reducing sugars with the N-terminus of free amino acids of proteins. Nucleic acids and lipids can also be modified. This reaction results in the reversible formation of a Schiff base and after structural rearrangements, more stable but still reversible Amadori products are formed. Finally, a fraction of Amadori products are further irreversibly modified to form AGEs[49,50]. Due to the slow nature of this reaction, AGE formation via the Maillard reaction can take months or even years. This type of AGE formation is predominantly present in long-lived proteins like extracellular matrix proteins such as collagen. Skin and vascular stiffening, and modification of lens proteins in eye disease are common manifestations of AGE formation via the classical Maillard reaction on long-lived proteins[51,52].

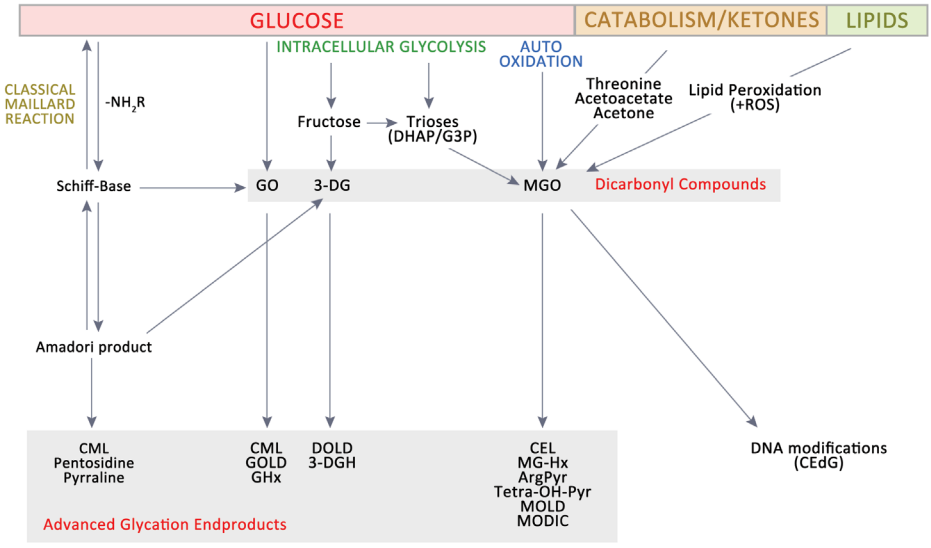

Figure 1.3 A diagram of the formation of dicarbonyls and AGEs. AGE formation is depicted via the classic Maillard reaction, and via fast glycation reactions with dicarbonyl compounds. Dicarbonyls can be formed in several ways: directly from glucose and fructose, from triose intermediates during glycolysis, during catabolism processes, from ketone bodies or during ketosis, from lipid peroxidation, and from Maillard reaction intermediates (Schiff bases and Amadori products). After reactions with specific amino acids (or DNA), a broad range of AGEs can be formed.

Dicarbonyl compounds

Dicarbonyl compounds are highly reactive glycating agents; more potent than glucose itself, they are the major source of AGE formation in the body[53]. In fact, the intracellular formation of AGEs via dicarbonyls is more abundant than the formation via the Maillard reaction. Dicarbonyls are most often characterized as harmful biomolecules that are progressively formed due to hyperactivity of the glycolytic pathway. In our research, we focussed on the dicarbonyls MGO, glyoxal (GO), and 3-deoxyglucosone (3-DG). Dicarbonyls can be formed in several ways: directly from glucose and fructose, from triose intermediates during glycolysis, during catabolism processes, from ketone bodies or during ketosis, from lipid peroxidation, and from Maillard reaction intermediates as described below. An overview of this process is depicted in Figure 1.3. Specific dicarbonyl compounds react with different amino acids and protein residues to form a wide variety of AGEs. MGO for example reacts with arginine residues to form the Nδ-(5_hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1)[54], as well as with lysine residues, to form Nε-(carboxyethyl)lysine (CEL)[55]. The modification of DNA is another kind of non-enzymatic glycation by MGO. Examples are N^2-(1-carboxyethyl)-2'-deoxyguanosine (CEdG)[56] and 3-(2′-deoxyribosyl)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b]purin-9(8)one (MGdG)[57]. The glycation of DNA can result in DNA instability, transversions, and crosslinking[57-59]. Glyoxal and 3-DG can react with arginine and lysine to form Nε-(carboxymethyl)lysine (CML) and pyrraline, respectively[49,60].
In this thesis, we analyzed plasma and urine samples for the dicarbonyls MGO, GO, and 3-DG, and the AGEs CML, CEL, and MG-H1, in their free- and protein-bound form. Furthermore, protein-bound pentosidine and skin-autofluorescence measurements were performed, as a measure for cross-linking and AGE accumulation in the skin, respectively.

Pathophysiology of methylglyoxal

Although MGO is associated to several pathological effects via protein glycation, some pathophysiological consequences can also be directly attributed to increased MGO levels.
MGO stress can be caused by increased production of MGO, or by reduced detoxification. MGO can be enzymatically detoxified by the glyoxalase system, which is present in the cytoplasm of all cells. Glyoxalase 1 (GLO1) converts MGO to S-D-lactoylglutathione, which in turn is converted to D-lactate by glyoxalase 2 (GLO2). Under healthy conditions, and in the presence of GSH, almost all of the unbound MGO is metabolized via this glyoxalase pathway[61,62].

Recent reviews give a complete description of the pathophysiology of MGO[63,64], which is briefly discussed below, with a focus on the endothelium.

MGO accumulation is associated with endothelial dysfunction and damage to the microcirculation. In cell and animal models, GLO1 overexpression improved NO-mediated relaxation[65], and prevents endothelial dysfunction[66] and hyperglycemia-induced AGE formation[67]. Elevated MGO concentrations have been shown to impair the endothelium[68] and microcirculation[69]. One mechanism of endothelial damage is via induced (mitochondrial) oxidative stress[70]. The overproduction of superoxide by the mitochondria during hyperglycemia, leads to higher levels of reactive oxygen species (ROS), which are implicated in the formation of dicarbonyl stress, NFkB activation, lipid peroxidation, and the creation of a pro-inflammatory environment[71]. Increased superoxide production and disturbed nitric oxide synthase metabolism[70,72] in endothelial cells, and disrupted vasoregulation in smooth muscle cells[73] can all contribute to MGO-induced endothelial dysfunction. Not just endothelial cells, but microvascular organs in general are susceptible to MGO toxicity. Many preclinical and clinical studies have found associations between MGO and diabetic nephropathy, retinopathy, and neuropathy[63,64].

MGO is also strongly associated with insulin resistance and obesity. Direct MGO modifications and alterations in signalling pathways have different negative effects on insulin metabolism in cell and animal models[74-76]. The association between MGO and insulin resistance has also been established in a human cohort[77]. In obesity, under hypoxic conditions and during increased inflammation, GLO1 activity can also be reduced[78,79], resulting in strongly increased levels of MGO.

MGO is also involved in the development of macrovascular complications; it can increase atherosclerosis[80], and is associated with intima-media thickening in diabetic individuals[81]. MGO is able to modify LDL[82,83] and HDL[84] resulting in increased atherogenic LDL particles and reduced anti-inflammatory effects of HDL. Studies regarding ischemia induced vascular damage show that MGO also hampers defensive and repair mechanisms after ischemic damage, which can be reduced by overexpression of GLO1[85].

Recently plasma MGO has been associated with incident cardiovascular disease and mortality in type 1 and type 2 diabetes[86,87], which underlines the clinical relevance of interventions in the glycation pathway.

Pathophysiology of AGEs

A large part of MGO (and dicarbonyl) pathophysiology is explained by structural and functional modifications of proteins. Irreversible glycation of proteins results in the formation of AGEs. AGE accumulation is associated with a natural ageing process, however this process is also strongly increased in obesity or diabetes[88-90]. Prolonged

hyperglycemia and hypertrophic adipose tissue can drastically increase the endogenous formation of diacarbonyls and AGEs.
The pathophysiological effects of AGEs are abundant, and are often categorized in three groups[46]: extracellular, intracellular, and effects partly mediated by receptor binding (Figure 1.4).

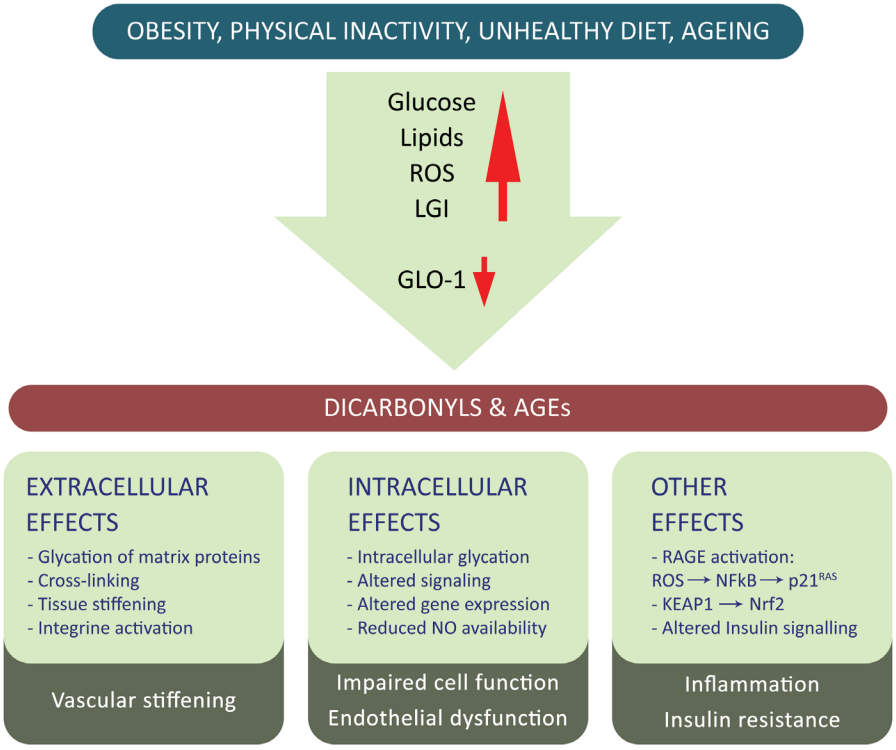

Figure 1.4 Pathophysiological effects of dicarbonyls and AGEs can be categorized into three main groups: extracellular effects like tissue stiffening, intracellular effects like altered cell signalling and gene expression, and thirdly other effects can be mediated by the AGE receptor or Nrf-2.

Dicarbonyls can modify proteins in the extracellular matrix. Protein modifications in the extracellular matrix are an important consequence of glycation, especially in older individuals, as these modifications accumulate with age. Collagen cross-links like pentosidine and glucosepan can result in decreased elasticity of blood vessels leading to arterial stiffening[52], but also to diabetic retinopathy[91]. Matrix structure, permeability and also integrin binding can be disturbed by this process[92,93]. Vascular stiffening, tendon stiffening, glycation of lens proteins, and reduced glomerular function are all examples

of glycation effects on the extracellular matrix[94,95]. Glycation of cell-matrix structures has also been associated with reduced adhesion of endothelial cells to the extracellular matrix[96].
Fast non-enzymatic AGE formation also occurs intracellularly via the rapid formation of dicarbonyl compounds. Endothelial cells are especially susceptible to this process as they take up glucose in a concentration dependent manner. Dicarbonyls can modify intracellular proteins with the formation of AGEs, and can cause functional abnormalities and activation of intracellular signaling pathways[46,53,67]. Reduced endothelial nitric oxide synthase (eNOS) expression and quenching of nitric oxide are examples of glycation effects in endothelial cells[97,98]. Glycation of insulin and the insulin receptor is another route by which glycation can affect the insulin signaling pathways[74,99]. Impaired insulin signaling and insulin mediated glucose uptake by MGO are important findings that require further evaluation.

A third pathway of cellular damage as induced by the glycation pathway is mainly via binding to the AGE receptor (RAGE)[100]. RAGE is expressed on several cell types including lymphocytes, endothelial cells and smooth muscle cells[101]. Via RAGE binding and consequent increased levels of ROS, the nuclear transcription factor κB (NFκB) pathway can be activated, which in turn results in the expression of pro-inflammatory cytokines and growth factors, contributing to diabetic complications[102-104]. Besides RAGE, several other AGE receptors have been described that are associated with AGE levels[105,106]. So far, these receptors are no primary target for anti-glycation interventions.
A final note, not all effects of MGO or AGE are necessarily harmful. An important effect of MGO to investigate is the stimulation of the Kelch-like ECH-associated protein 1 (KEAP1) – nuclear factor erythroid 2-related factor 2 (Nrf-2) axis[107] by MGO. Nrf-2 regulates oxidative stress responses, and is an important cytoprotective mechanism against oxidative damage[108].

III. Current interventions in the glycation pathway

Lifestyle interventions

Both dietary weight loss and increased physical activity are fundamental strategies to improve metabolic and vascular health. Postprandial elevations of glucose and dicarbonyls are an important target in the prevention of complications, and both energy restriction and lifelong exercise have shown the potential to lower dicarbonyl stress[109,110]. A low caloric diet furthermore showed decreased serum AGEs in overweight and obese adults[111]. Other dietary intervention strategies in the glycation pathway

include the use of AGE-restricted diets. Several studies have investigated the biological effects of a reduction in dietary intake of AGEs and dicarbonyls. While some studies describe positive effects on inflammation and insulin sensitivity[112,113] a large clinical trial from our department recently showed no effects on glucose metabolism or vascular function[114]. In chapter 2 and 3, we describe two lifestyle interventions studies with dietary weight loss and physical activity, and investigate whether they affect dicarbonyls and glycation markers as measured with state-of-the art analytical methods.

Scavenging of dicarbonyls

Many strategies focus on the reduction of endogenous formation of AGEs, predominantly via scavenging of MGO and other dicarbonyls.

Aminoguanidine (also called pimagedine) is a well-known inhibitor of AGE formation, and has shown to prevent diabetic complications in experimental studies. Its functionality is based on scavenging dicarbonyl compounds[115]. Because of little clinical effects in human trials and potential safety concerns, further studies with aminoguanidine as an anti-glycation therapy have been terminated[116].

Alagebrium has shown great promise as an inhibitor of glycation[117,118]. Alagebrium has initially been described as a cross-link breaker[118], but new evidence indicates that it functions as a MGO scavenger, thus reducing AGE formation[89]. While improvements in cardiovascular health markers have been reported in hypertensive individuals in an RCT[119], a second trial showed no beneficial results[120]. Current investigations on alagebrium are terminated, because of practical obstacles.

L-carnosine and its derivative carnosinol have also been described as natural agents able to quench dicarbonyls, prevent glycation, and improve glucose metabolism[121-123]. Future trials with carnosinol are needed to evaluate its therapeutic effect in humans.

Arginine-containing peptides could also serve as MGO scavengers since MGO has a high affinity for binding arginine residues[124]. However, high efficacy in clinical trials is questionable because of the very high concentrations of endogenous arginine.

Many different polyphenolic and flavonoids compounds including quercetin, epicatechin, resveratrol, and hesperidin have been demonstrated to have MGO scavenging abilities[125]. The first two are described in chapter 4 of this thesis. While data from clinical trials are limited, several polyphenolic compounds have shown great potential in experimental work. Very compelling data from a resveratrol and hesperidin study focusses on the activation of GLO1, as described below[126]. The polyphenol curcumin and the heterotricyclic compound monascin (monascoflavin) are also examples of natural compounds targeting glycation, in this case by functioning as peroxisome proliferator-activated receptor γ (PPARγ) agonists. Both substances have shown to

elevate PPARγ activity and could potentially attenuate oxidative stress and reduce RAGE expression[127,128].
Last but not least, pyridoxamine has been described as a multifunctional pharmaceutical, able to target dicarbonyl stress[129]. Our group and several others have shown promising results in animal studies[130]. Further details are described in chapters 6 to 8 of this thesis. Pyridoxamine has also been investigated in the setting of nephropathy with moderate results[131,132].

GLO1 inducers

Natural detoxification via GLO1 is an important strategy with several functional candidates. Several polyphenols like resveratrol and fisetin are known to upregulate GLO1 expression[133,134]. Similarly, sulforaphane and candesartan have both shown to upregulate GLO1 activity[135,136]. Mechanistically these compounds can activate Nrf2, which regulates oxidative stress responses via antioxidant responsive elements (ARE) and can in turn active ARE-dependent anti-oxidant enzymes. GLO1 has this ARE in its promotor region, resulting in increased GLO1 mRNA production as a results of Nrf2 stimulation[108,136]. Recently, a GLO1 inducer (a trans-resveratrol and hesperidin co-formulation) has been described that showed lower MGO concentrations, improved glycemic control, and vascular function in a clinical trial[126].

Anti-diabetic medication

The formation of AGEs is directly or indirectly derived from glucose and therefore medication improving glucose metabolism and reducing hyperglycemia is a way to reduce the formation of dicarbonyls and AGEs. Triose phosphates degradation during glycolysis is a major part of MGO formation. Improved glucose metabolism and a reduction in hyperglycemia could thus also be very effective in the reduction of MGO formation.
Metformin is an oral glucose-lowering therapeutic, able to suppress hepatic gluconeogenesis and increasing cellular glucose uptake[137]. It has shown to reduce MGO by direct scavenging MGO, and/or via an upregulation of GLO-1[138-140]. Pioglitazone, like metformin, is mainly used as a glucose-lowering medication. Pioglitazone can also downregulate RAGE expression and inhibit ROS production and NFκB activation, via the activation of PPARγ[141].

Knowledge gaps

Several large cohort studies have established the association between dicarbonyl stress (mainly MGO), AGE accumulation, and diabetic complications. Furthermore, experimental studies have greatly increased our knowledge of the pathophysiological pathways activated or stimulated by dicarbonyl compounds and AGEs. However, there are no clear-cut specific intervention strategies available at this moment. Studies so far lack state-of-the-art analytical techniques for the analysis of dicarbonyls and AGEs, others lack pure pharmaco-nutritional supplements, and a few studies were terminated due to a lack of effectivity or due to safety concerns.

Our aim is to investigate lifestyle interventions, like dietary weight loss and physical activity, and pharmaco-nutritional strategies as potential new intervention strategies to reduce dicarbonyls and AGEs, or counter their effects.

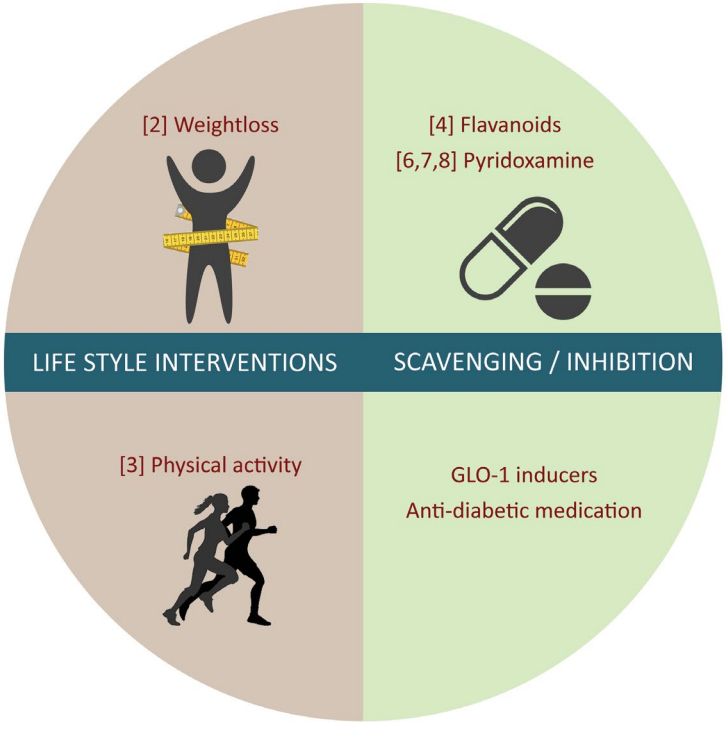

Figure 1.5 Short overview of the intervention strategies investigated in this thesis. The numbers refer to the specific chapters in the thesis. Other intervention strategies, like GLO1 inducers or the effect of anti-diabetic medication are not investigated but are also a part of inhibitory strategies in the glycation pathway.

Overview of this thesis

In chapter 2, we studied the effects of a weight loss intervention on glycation markers. Weight loss by caloric restriction might be considered as the most significant (or definitely the most studied) 'lifestyle' intervention in the prevention of chronic metabolic diseases and CVD. The weight loss intervention therefore also serves as a hallmark in this thesis as it can serve as a reference point for other (nutraceutical) interventions in the glycation pathway. We evaluated a large array of dicarbonyls and AGE compounds and were able to compare data before and after weight reduction with that of lean individuals. Furthermore, we also investigated the effect of weight loss on tissue glycation as estimated by skin autofluorescence. In chapter 3, we described the effects of physical activity and a high intensity interval training (HIIT) intervention on glycation markers in an ageing population. We compared dicarbonyl and AGE concentrations between three groups of ageing individuals with different levels of physical activity. Furthermore, we assessed the effect of a HIIT in individuals from the highest risk category on glycation levels. In chapter 4, we investigated the effects of pure flavonoids epicatechin and quercetin on dicarbonyls and AGEs. We assessed the possible MGO scavenging capacity of these flavonoids and their effect on GLO1. In chapter 5-8 we focused on pyridoxamine. In chapter 5 we described the background and methodological context of a clinical trial with pyridoxamine. In chapter 6, we described the metabolization of pyridoxamine and its potential as a novel treatment in vitamin B6 deficiency and in the prevention of chronic metabolic disease. Finally, in chapter 7 and 8 we presented the outcome of our clinical trial with pyridoxamine. Pyridoxamine has now for the first time been evaluated in an RCT with regard to glycation, insulin sensitivity, inflammation, micro-, and macrovascular health. Primary outcomes, metabolic markers, glycation markers, insulin and glucose metabolism, and the effect on the microcirculation have been described in chapter 7. In chapter 8, we reported all the macrovascular endpoints. Finally, chapter 9 ends with a summary and general discussing on the abovementioned interventions and an impact statement on this thesis.

Table 1.1 Interventions to reduce dicarbonyl compound and ages.

Intervention Type	**Effect**	**Examples** (Chapters from this thesis and other studies)
1. Lifestyle interventions	Dietary weight loss and increased physical activity to reduce formation	Weight loss by caloric restriction (Chapter 2) Physical activity/exercise training (Chapter 3)
2. Scavenging or inhibition of endogenous formation	Pharma/nutraceuticals to reduce circulating levels of dicarbonyls and AGEs	Flavonoids: quercetin, epicatechin (Chapter 4) Pyridoxamine (Chapter 6-8) Aminoguanidine Alagebrium L-carnosine/carnosinol Arginine-peptides
3. Detoxification by the glyoxalase system	Pharma/nutraceuticals to induce GLO-1 activity	GLO-1 inducer (trans-resveratrol & hesperidin) Sulforaphane
4. Anti-diabetic & anti-hyperglycemic medication	Medication improving glucose metabolism to reduce formation	Metformin Benfotiamine/Thiamine Pioglitazone
5. Dietary interventions*	Reduction of exogenous intake of dicarbonyls and/or AGEs	Low AGE diet Low MGO diet

This table shows an overview of interventions in the glycation pathway with a proposed mechanism and several examples of each category. *These dietary interventions are focused on a reduction of exogenous (dietary) intake of AGEs or MGO. All other intervention focus on a reduction of endogenous dicarbonyls and AGEs.

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Chapter 2

Diet-induced weight loss reduces postprandial dicarbonyl stress in abdominally obese men: secondary analysis of a randomized controlled trial

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Abstract

Aims

Dicarbonyl compounds contribute to the formation of advanced glycation endproducts (AGEs) and the development of insulin resistance and vascular complications. Dicarbonyl stress may already be detrimental in obesity. We evaluated whether diet-induced weight loss can effectively reverse dicarbonyl stress in abdominally obese men.

Materials and methods

Plasma samples were collected from lean (*n*=25) and abdominally obese men (*n*=52) in the fasting state, and during a mixed meal test (MMT). Abdominally obese men were randomized to 8 weeks of dietary weight loss or habitual diet, followed by a second MMT. The α-dicarbonyls methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG) and AGEs were measured by UPLC-MS/MS. Skin autofluorescence (SAF) was measured using the AGE reader. T-tests were used for the cross-sectional analysis and ANCOVA to assess the treatment effect.

Results

Postprandial glucose, MGO and 3-DG concentrations were higher in obese men as compared to lean men ($p<0.05$ for all). Fasting dicarbonyls, AGEs, and SAF were not different between lean and obese men. After the weight loss intervention, fasting MGO levels tended to decrease by 25 nmol/L (95%-CI: -51-0.5; p=0.054). Postprandial dicarbonyls were decreased after weight loss as compared to the control group: iAUC of MGO decreased by 57% (5280 nmol/L·min; 95%-CI: 33-10526; p=0.049), of GO by 66% (11329 nmol/L·min; 95%-CI: 495-22162; p=0.041), and of 3-DG by 45% (20175 nmol/L·min; 95%-CI: 5351-35000; p=0.009). AGEs and SAF did not change significantly after weight loss.

Conclusion

Abdominal obesity is characterized by increased postprandial dicarbonyl stress, which can be reduced by a weight loss intervention.

Introduction

The ever-increasing rise in obesity contributes to the increasing incidence of type 2 diabetes and cardiovascular disease[1]. The formation of dicarbonyl compounds in obesity may be a key driver in the development of type 2 diabetes and cardiovascular disease[2]. Dicarbonyl compounds are reactive metabolites that are mainly formed as a byproduct of glycolysis, especially in the postprandial state. Dicarbonyl compounds may react with protein residues to form advanced glycation endproducts (AGEs) which are linked to the detrimental effects on cellular and vascular function[3,4]. In addition, in animal models of obesity, increased levels of dicarbonyls are linked to adipose tissue dysregulation[5,6].
Methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG) are the major dicarbonyl compounds[7]. Recently, we demonstrated that individuals with type 2 diabetes had higher plasma levels of MGO, GO and 3DG during an oral glucose load[8] or a meal[9] compared to healthy individuals. As MGO is the major precursor in the formation of AGEs, it is a key player in the development of age-related diseases[10]. Moreover, MGO has been associated with the development of diabetic complications[3], hypertension[11], atherosclerosis[12], cancer[13], and neurodegenerative disorders[14]. In addition, we recently found MGO to be associated with incident cardiovascular disease in both type 1 and type 2 diabetes[15,16]. The glyoxalase system, with glyoxalase I (GLO1) as the rate-limiting enzyme, is responsible for the enzymatic detoxification of MGO. It has been demonstrated that GLO1 expression and activity is decreased in adipose tissue and liver in animal models of obesity[5,6]. Downregulation of GLO1, as well as increased MGO formation may contribute to dicarbonyl stress in obesity and the health impairment of the obese phenotype.
In obese women with type 2 diabetes we demonstrated that postprandial dicarbonyl stress could be reduced by a very-low calorie diet[9]. As we hypothesize that dicarbonyl stress is an early contributor to incident T2D and its (cardiovascular) complication, we thus investigated the effect of a dietary weight loss intervention on dicarbonyl stress and AGEs in abdominally obese individuals. We addressed this in two ways. First, we investigated whether abdominally obese individuals had higher fasting and postprandial plasma levels of dicarbonyls and AGEs, as well as skin autofluorescence (SAF). The latter is considered to be a reflection of tissue AGEs. Second, we investigated whether a dietary weight loss intervention could reduce plasma levels of dicarbonyls and AGEs, as well as SAF.

Materials and methods

Study population

The methods of this randomized controlled trial have been described in detail previously[17-19]. Briefly, we included 52 abdominally obese men, as well as 25 lean men. All 74 individuals were between 18 and 65 years, nonsmoking, nondiabetic, and without cardiovascular disease. A stable body weight (weight difference <3 kg for ≥3 months) was required, and inclusion was based on waist circumference <94 cm for the lean men and between 102 and 110 cm for abdominally obese men.

Additional exclusion criteria were: fasting plasma glucose >7.0 mmol/L, HbA1c >6.5%, serum total cholesterol >8.0 mmol/L, serum triglycerides >4.5 mmol/L, systolic blood pressure >160 mmHg. Contraindications for MRI were not allowed and neither was medication use affecting glucose metabolism, blood pressure, or serum lipids.

The study was conducted in accordance with the Declaration of Helsinki, approved by the Ethics Committee of Maastricht University Medical Centre, and registered at ClinicalTrials.gov (NCT01675401). Written informed consent was obtained from all participants before study enrollment.

Study design and intervention

Abdominally obese men were randomly assigned to either an 8-week weight loss program or maintaining their habitual diet for 8 weeks. Measurements took place before and after the 8-week period. Lean individuals were studied at baseline only.

Individuals assigned to the weight-loss program consumed a very-low-calorie diet (VLCD; Modifast; Novartis Nutrition, The Netherlands) providing 2.1 MJ (500 kcal) per day for 4 to 5 weeks. Hereafter, a mixed-solid, calorie-restricted diet, which provided 4.2 MJ/day for 1 to 2 weeks, was prescribed as a transition period. The composition of this diet was in agreement with Dutch dietary guidelines, and was described in detail earlier[17]. In weeks 7 and 8, individuals consumed daily menus based on the estimated energy requirements based on their newly achieved body weight in order to retain a stable weight.

Individuals assigned to the weight-stable control group were asked to maintain their habitual diet throughout the study period and were monitored to avoid fluctuations in weight.

In addition, individuals from all groups were instructed not to change their physical activity levels and write down any protocol deviations in a daily diary. The day before the measurements, individuals were asked not to consume high-fat foods or alcohol or to perform any strenuous physical exercise. After a 12-hour overnight fast, volunteers

arrived at the research facilities by public transport or by car to standardize pre-measurements conditions. All measurements were conducted in a temperature-controlled room at 24±0.5°C.

Mixed meal test

A mixed meal test was performed at baseline and after the intervention period. After an acclimatization period and the placement of an intravenous cannula, a fasting venous blood sample was drawn. Within ten minutes, all participants had to consume a standardized mixed meal. Blood samples were collected at regular intervals as described previously[19]. Samples were analyzed for glucose and dicarbonyl postprandial effects, at 0 min (fasted), 60 min, 120 min, and 240 min after consumption of the mixed meal.
The mixed meal consisted of two muffins and 300 mL low-fat milk (0% fat milk; Friesland-Campina, Woerden, The Netherlands). The mixed meal had an energy content of 1100 kcal and provided 26.5 g protein (9.6 En%), 121.0 g carbohydrates (44 En%) and 56.6 g fat (46.6 En%), as previously described[19].
For the plasma dicarbonyls MGO, GO, 3-DG, and for the glucose levels measured during the mixed meal test, we calculated the incremental area under the curve (iAUC) using the trapezoidal rule[20]. To compare the postprandial changes in plasma dicarbonyl or glucose levels, the incremental area under the curve (iAUC) was calculated by subtracting the respective fasting levels from each individual data point. The iAUC of the dicarbonyl levels is expressed as nmol/l x min and the iAUC for glucose is expressed as mmol/l x min.

Baseline characteristics

Plasma glucose concentrations were determined with a YSI2300 glucose analyzer (YSI). Serum fasting samples were analyzed for total cholesterol (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany), HDL cholesterol (precipitation method; Roche Diagnostics, Mannheim, Germany), triacylglycerol with correction for free glycerol (GPO Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA), HbA1c (Bio-Rad), and insulin (RIA; Millipore, Billerica, MA, USA) concentrations. Twenty-four-hour ambulatory blood pressure measurements were performed (Mobil-O-Graph; I.E.M.). Blood pressure was recorded every 15 min during daytime and every 30 min at night. Subjects were asked not to change their daily activities during this period except the avoidance of intensive physical exercise. From these recordings 24-h mean ambulatory blood pressure was calculated.

Measurement of plasma dicarbonyls, AGEs and skin autofluorescence

EDTA and NaF tubes were centrifuged at 1300 × g for 15 min at 4°C to obtain plasma. The supernatants were transferred into 1.5 ml Eppendorf tubes and stored at −80°C until further analysis. Plasma glucose concentrations were measured in NaF-plasma (Horiba ABX, Montpellier, France). Plasma levels of dicarbonyls and AGEs were measured in EDTA plasma samples.

Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to determine the plasma levels for MGO, GO and 3-DG, as previously described[21]. Plasma levels of the free and protein-bound AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(1-carboxyethyl)lysine (CEL), and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), were determined with UPLC-MS/MS. Protein-bound pentosidine was determined with high performance liquid chromatography with fluorescent detection[22]. Coefficients of variation for all MS/MS analyses were below 10%.

Prior to each mixed meal test, skin autofluorescence (SAF) was measured using the AGE reader (DiagnOptics Technologies, Groningen, The Netherlands). The AGE reader is a device that uses the fluorescent properties of certain protein modifications and AGEs, to estimate their level of accumulation in the skin. Technical details have been described elsewhere[23,24].

Measurement of ketone bodies

Serum samples were collected at the start and end of the intervention period. Ketone body concentrations were measured using nuclear magnetic resonance (NMR). Details about this analysis have been described in detail previously[25,26].

Measurement of GLO1, AKR1B1, and ALDH2

PBMC's were isolated from the blood samples drawn in the fasted state at baseline and after the 8-week period by using BD Vacutainer Cell Preparation Tubes. RNA was isolated (RNeasy Micro kit, Qiagen, Venlo, The Netherlands) and quantified (Nanodrop ND 1000, Nanodrop Technologies, Wilmington, DE, USA); an Agilent 2100 Bioanalyser with RNA 6000 microchips was used to check integrity. Samples were included for microarray analysis if the RNA integrity number was >7. PBMC samples from 15 lean and 29 abdominally obese individuals) yielded enough RNA of sufficient quality to perform microarray analysis. The expression of glyoxalase 1 (GLO1), aldo-keto reductase family 1 member B (AKR1B1), and aldehyde dehydrogenase (ALDH2) between groups was tested with an independent samples t-test with empirical Bayes correction. Details regarding the microarray analysis have been described elsewhere[25].

Statistics

Normally distributed variables are presented as means ± standard deviations or as median with interquartile range, where appropriate. Cross-sectional differences between lean and abdominally obese men, were analyzed by an independent Student's *t*-test. Treatment effects of the weight loss intervention compared to the weight stable control group were analyzed by one-way ANCOVA with adjustment for baseline values. Differences in postprandial changes between the study groups were analyzed using mixed ANOVA with intervention or obesity as between-subjects factor and time within-subjects factor. For the mixed ANOVA intervention analysis, we subtracted the baseline postprandial curve from the postprandial curve at follow-up. Non-normally distributed values were log transformed prior to analysis. A p value ≤0.05 was considered to be significant. All statistical analyses were performed with IBM SPSS Statistics Software version 20.0.

Results

A CONSORT flow diagram of the intervention design and inclusion is shown in Supplemental Figure S2.1. At baseline, 25 normal-weight and 52 abdominally obese participants were analyzed. From the abdominally obese participants, 49 men completed the study and were included in the analyses; 23 in the weight loss intervention and 26 in the control group[18].

The baseline characteristics are shown in Table 2.1. Though age was not different between the subgroups, most anthropometric and metabolic indices were significantly different between the lean and obese groups (Table 2.1). Further details have been described previously[18,19].

Table 2.1 Study population characteristics.

	Lean (n=25)	Obese (n=52)	Weight Loss Baseline (n=23)	Weight Stable Baseline (n=26)
Age [Years]	53.7 [25.0 - 61.6]	51.8 [45.7 - 60.7]	52.4 [46.8 - 61.7]	52.0 [45.4 - 61.1]
Body weight [kg]	74.9 ± 8.3	96.9 ± 8.4 ***	98.2 ± 8.1	95.9 ± 8.9
Fat Free Mass [kg]	60.8 ± 6.4	69.6 ± 6.1 ***	69.7 ± 5.9	69.2 ± 6.6
Fat Mass [kg]	14.1 ± 4.8	27.2 ± 4.6 ***	28.5 ± 4.2	26.7 ± 4.6
BMI [kg/m^2]	23.3 ± 1.8	30.1 ± 2.1 ***	30.2 ± 1.5	29.9 ± 2.5
Waist Circumference [cm]	84.9 ± 6.3	106.5 ± 3.6 ***	106.8 ± 3.4	106.2 ± 3.8
Hip Circumference [cm]	96.6 ± 4.3	107.5 ± 5.2 ***	108.1 ± 4.4	107.2 ± 6.0
HOMA-IR	1.65 ± 0.46	2.84 ± 1.38 ***	2.64 ± 1.21	2.90 ± 1.40
Fasting Plasma Glucose [mmol/L]	5.35 ± 0.29	5.64 ± 0.48 **	5.49 ± 0.37	5.75 ± 0.53
Fasting Plasma Insulin [mIU/L]	7.1 ± 1.9	11.6 ± 5.4 ***	11.2 ± 5.0	11.5 ± 5.6
HbA1c [%]	5.18 ± 0.37	5.30 ± 0.37	5.24 ± 0.35	5.33 ± 0.39
Total Cholesterol [mmol/L]	4.55 ± 0.78	5.56 ± 0.97 ***	5.60 ± 1.09	5.63 ± 0.86
LDL-Cholesterol [mmol/L] †	2.82 ± 0.70	3.68 ± 0.89 ***	3.70 ± 0.96	3.71 ± 0.85
HDL-Cholesterol [mmol/L]	1.26 ± 0.26	1.11 ± 0.21 **	1.15 ± 0.17	1.08 ± 0.23
Triacylglycerol [mmol/L]	0.95 [0.67 - 1.11]	1.66 [1.17 - 2.19] ***	1.43 [0.94 - 2.02]	1.75 [1.20 - 2.38]
24-h Systolic BP [mmHg]	117.5 ± 8.8	123.4 ± 8.7 **	120.2 ± 9.2	125.6 ± 7.6
24-h Diastolic BP [mmHg]	72.5 ± 9.4	80.4 ± 7.3 ***	78.3 ± 7.6	82.0 ± 6.7

Data presented as mean ± SD or median [IQR]. Baseline differences between obese and lean men were assessed by means of independent Student's T-test or Mann-Whitney U test where appropriate; *p<0.05, **p<0.01, ***p<0.001 compared to baseline values of the lean men. (†) LDL Cholesterol analyzed in 25 lean and 50 obese men [18].

Cross-sectional analysis

In the fasting state, the dicarbonyls MGO, GO, and 3-DG were not significantly different between lean and abdominally obese men (Table 2.2). We found lower levels of protein-bound CML in the abdominally obese men compared to lean men (p=0.025). Plasma concentrations of protein-bound CEL, protein-bound MG-H1 and pentosidine, and free CML, CEL and MG-H1, did not show a significant difference between lean and obese men (Table 2.2). SAF was not different between lean and obese individuals (Table 2.2).

Postprandial concentrations of glucose, MGO and 3-DG, as assessed by the iAUC during the mixed meal test, were significantly higher in obese compared to lean men (Figure 2.1, Table S2.3). Results from the mixed ANOVA analyses of the mixed meal data showed that postprandial increases in MGO (p=0.039) and 3-DG (p=0.001) were significantly different between lean and obese individuals, this was not the case for GO concentrations (p=0.29).

Two hours after the mixed meal, glucose (lean 5.87±0.91 mmol/L vs. obese 6.78±1.10 mmol/L, p=0.001), MGO (lean 321±51 nmol/L vs obese 357±70 nmol/L, p=0.025) and 3-DG (lean 1064±163 nmol/L vs. obese 1238±203 nmol/L, p<0.001)

concentrations were higher in obese compared to lean men; there was no significant difference after four hours.

Table 2.2 Cross-sectional analysis of fasting dicarbonyls, AGEs, SAF, and postprandial dicarbonyls.

	Lean (n=25)	Obese (n=52)	P value
Fasting concentrations			
MGO (nmol/L)	293 ± 52	297 ± 54	0.75
GO (nmol/L)	755 ± 195	686 ± 139	0.079
3-DG (nmol/L)	958 ± 74	993 ± 117	0.17
CML (nmol/L)	94 [81-115]	96 [83-114]	0.84
CEL (nmol/L)	48 [44-60]	53 [46-69]	0.12
MG-H1 (nmol/L)	130 [87-179]	124 [94-182]	0.34
Protein-bound CML (nmol/L)	81 [71-91]	74 [66-84]	0.025*
Protein-bound CEL (nmol/L)	31 [25-40]	35 [28-51]	0.059
Protein-bound MG-H1 (nmol/L)	241 [223-278]	237 [207-269]	0.47
Pentosidine (nmol/lysine)	0.40 [0.34-0.50]	0.35 [0.30-0.43]	0.29
SAF (Skin autofluorescence)	1.96 ± 0.40	2.08 ± 0.45	0.25

Data are presented as mean ± SD or median [IQR]. Baseline differences between obese and lean men were assessed by means of an independent samples t-test (*p<0.05, **p<0.01). Skewed variables (free and protein-bound AGEs) were Log transformed prior to analysis.

Dicarbonyls: MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone

Advanced glycation end products (AGEs): CML: N^{ε}-(carboxymethyl)lysine, CEL: N^{ε}-(1-carboxyethyl)lysine, MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Effect of the weight loss intervention

After an 8-week dietary weight loss intervention, BMI decreased by 10% on average and multiple cardiometabolic variables were improved, as described previously[17-19]. Most notably, there was a decrease in fasting plasma glucose of 0.24 mmol/L (95%-CI: 0.06-0.42; p<0.05), a decrease in HOMA-IR of 1.11 (95%-CI: 0.66-1.56; p<0.001), and a significant increase in whole body glucose disposal of 1.36 mg/kg/min (95%-CI: 0.81-1.92; p<0.001).

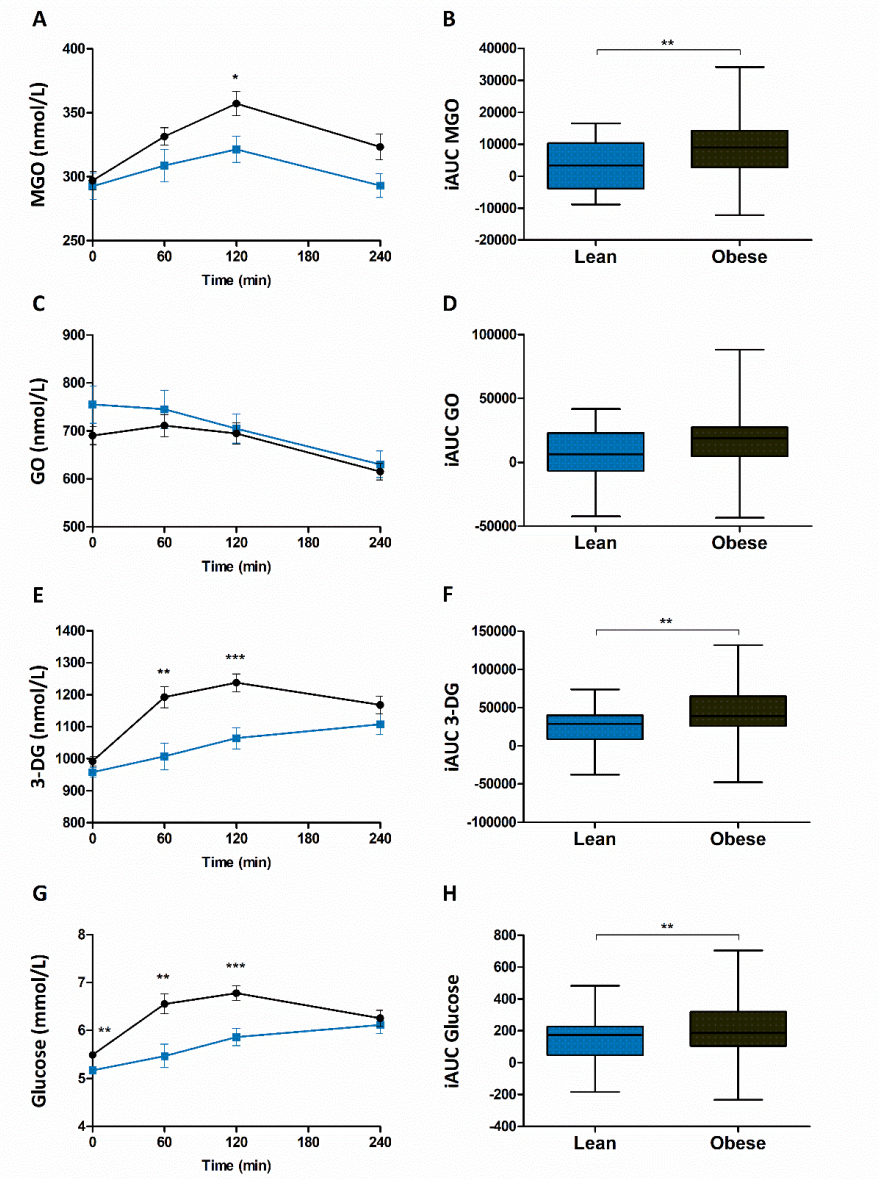

Figure 2.1 Cross-sectional analysis of postprandial dicarbonyls and glucose.
Graphs (A-B) show MGO concentrations in nmol/L during the mixed meal test, and the respective iAUC data in nmol/L x min. (C-D) show the same for GO and (E-F) for 3-DG. (G-H) show glucose concentrations in mmol/L and the respective iAUC in mmol/L x min. Black circle = obese, blue square = lean. Graphs are presented as mean with SEM, boxplots indicate median, 25th and 75th percentile, and min to max whiskers. Differences between obese and lean men were assessed by means of an independent samples t-test. P-values on top of the curves show the difference between both groups per time point; p-values on the boxplots indicate the iAUC difference (*p≤0.05, **p≤0.01, ***p≤0.001). iAUC: incremental area under the curve, MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone. Data shown in Supplementary Table S2.3.

Fasting MGO, but not other dicarbonyls, tended to decrease after weight reduction compared to the weight stable control group by 25 nmol/L (95%-CI: -51-0.5; p=0.054; Table 2.3). SAF was unaffected by weight loss (p=0.9; Table 2.3). Similarly, fasting concentrations of plasma AGE in the free and protein-bound form were largely unaltered by the weight loss intervention (Table 2.3). Only protein-bound CEL levels were significantly reduced by weight loss as compared to the control group by 16 nmol/L (95%-CI: 4-28; p=0.002).

Postprandial dicarbonyl levels in the weight reduction group were reduced as compared to the weight stable group (Figure 2.2, Table S2.4). The postprandial iAUC of MGO decreased by 57% (5280 nmol/L x min; 95%-CI: 33-10526; p=0.049), the iAUC of GO decreased by 66% (11329 nmol/L x min; 95%-CI: 495-22162; p=0.041), and the iAUC of 3-DG decreased by 45% (20175 nmol/L x min; 95%-CI: 5351-35000; p=0.009). Results from the mixed ANOVA analyses of the intervention data showed that postprandial increases in MGO (p=0.007) and 3-DG (p=0.043) were significantly decreased after weight loss as compared to the weight stable group. This was not the case for postprandial GO concentrations (p=0.144).

Postprandial dicarbonyl iAUC levels were reduced by weight loss to values similar to those of the lean subgroup (Supplementary Figure S2.2). After adjustment for glucose iAUC levels, the treatment effect on postprandial dicarbonyls was strongly attenuated (Supplementary Table S2.5).

GLO1, AKR1B1, ALDH2, and ketone bodies

The expression of GLO1, AKR1B1, and ALDH2, i.e. enzymes potentially involved in the degradation of MGO, did not differ between lean and obese individual in this study (Supplementary Table S2.1). Furthermore, there was no change in GLO1, AKR1B1, and ALDH2 expression by the weight loss intervention.

Recently, the ketone body acetoacetate has been identified as a potential scavenger of MGO[27]. Serum concentrations of acetoacetate and β-hydroxybutyrate were not different between lean and obese individuals (Supplementary Table S2.2). β-hydroxybutyrate, but not acetoacetate, was significantly increased by weight loss as compared to the weight stable control group (Supplementary Table S2.2; median changes in the weight loss group and control group were +20.8 µmol/l and -8.0 µmol/l, respectively; p=0.044).

Table 2.3 Effect of weight loss on fasting dicarbonyls, AGEs, SAF, and postprandial dicarbonyls.

	Weight loss (n=23)		Weight stable (n=26)		Treatment effect	
	Baseline	Follow-up	Baseline	Follow-up	Mean change (95% CI)[†]	P value[‡]
Fasting concentrations						
MGO (nmol/L)	291 ± 52	275 ± 52	300 ± 55	303 ± 42	-25 (-51, 0.5)	0.05
GO (nmol/L)	674 ± 152	607 ± 136	700 ± 135	654 ± 140	-35 (-106, 36)	0.32
3-DG (nmol/L)	950 ± 105	938 ± 86	1022 ± 102	1023 ± 114	-37 (-85, 10)	0.12
CML (nmol/L)	95 [82-130]	129 [84-148]	96 [82-114]	103 [86-123]	24 (-1, 49)	0.09
CEL (nmol/L)	54 [46-69]	51 [40-76]	53 [47-72]	56 [47-71]	-1 (-17, 14)	0.48
MG-H1 (nmol/L)	138 [95-190]	130 [102-284]	129 [90-177]	136 [99-199]	55 (-37, 147)	0.52
Protein-bound CML (nmol/L)	80 [70-86]	77 [67-84]	73 [64-81]	75 [68-83]	-3 (-8, 3)	0.29
Protein-bound CEL (nmol/L)	39 [29-57]	30 [26-39]	34 [26-44]	44 [32-59]	-16 (-28, -4)	0.002**
Protein-bound MG-H1 (nmol/L)	235 [207-275]	245 [221-285]	242 [210-258]	241 [210-259]	21 (-8, 49)	0.17
Pentosidine (nmol/lysine)	0.38 [0.31-0.45]	0.42 [0.36-0.48]	0.33 [0.31-0.40]	0.33 [0.29-0.39]	0.02 (-0.07, 0.11)	0.36
SAF (Skin autofluorescence)	2.07 ± 0.43	2.10 ± 0.39	2.16 ± 0.44	2.16 ± 0.39	0.01 (-0.10, 0.11)	0.90

Data are presented as mean ± SD or median [IQR]. Differences in changes between the weight loss treatment and weight stable control treatment, were tested using one-way ANCOVA with adjustment for baseline values. [(†)]Adjusted mean changes (estimated marginal means) with 95% CI are given. [(‡)]All P values are obtained from the ANCOVA analysis with adjustment for baseline values (*p<0.05, **p<0.01). Skewed variables (free and protein-bound AGEs) were Log transformed prior to analysis.
Dicarbonyls: MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone.
Advanced glycation end products (AGEs): CML: N^{ε}-(carboxymethyl)lysine, CEL: N^{ε}-(1-carboxyethyl)lysine, MG-H1: N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

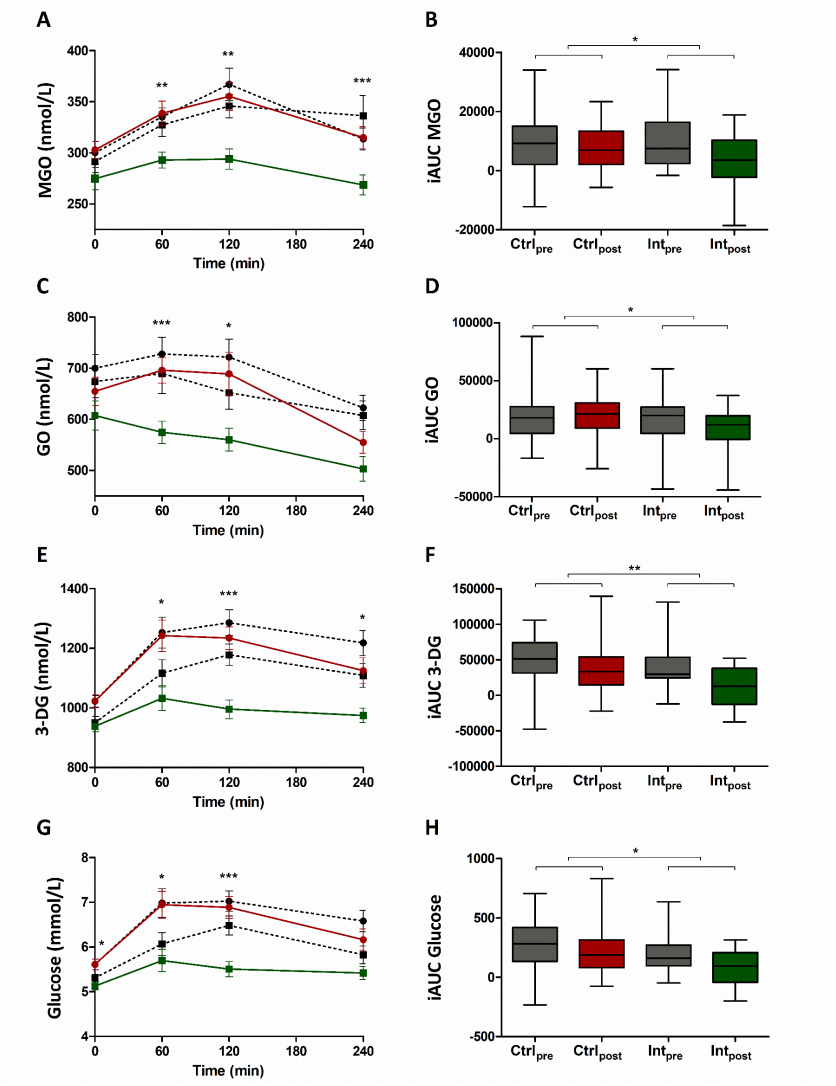

Figure 2.2 Effect of a weight loss intervention on postprandial dicarbonyls and glucose.
Graphs (A-B) show MGO concentrations in nmol/L during the mixed meal test, and the respective iAUC data in nmol/L x min. (C-D) show the same for GO and (E-F) for 3-DG. (G-H) show glucose concentrations in mmol/L and the respective iAUC in mmol/L x min. Black circle, dotted curve = $Ctrl_{pre}$ = control at baseline. Red cirlce = $Ctrl_{post}$ = control at follow-up. Black square, dotted curve = Int_{pre} = weight loss intervention at baseline. Green square = Int_{post} = weight loss intervention at follow-up. Graphs are presented as mean with SEM, boxplots indicate median, 25th and 75th percentile, and min to max whiskers. Differences in changes between the weight loss intervention and weight stable control were tested using one-way ANCOVA with adjustment for baseline values. P-values on top of the curves show the difference between both groups per time point; p-values on the boxplots indicate the iAUC difference (*p≤0.05, **p≤0.01, ***p≤0.001). iAUC: incremental area under the curve, MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone. Data shown in Supplementary Table S2.4

Discussion

We showed that in apparently healthy abdominally obese men, postprandial plasma dicarbonyl levels were significantly higher compared to lean men. This difference was not manifest in the fasting state. Furthermore, in the fasting state we found no significant difference in SAF and a panel of specific plasma AGEs between lean and obese individuals, except for protein-bound CML, which was lower in the abdominally obese subgroup compared to lean men.
Most importantly, a diet-induced weight loss intervention reduced postprandial dicarbonyl levels in abdominally obese men as compared to the control group. Fasting levels of MGO were marginally decreased after weight loss, but not 3DG, GO, AGEs, and SAF.

Cross-sectional analysis

We found a higher iAUC of postprandial dicarbonyls in abdominally obese men, compared to lean men. This observation is in line with our previous finding in obese women with type 2 diabetes[9]. However, we previously reported higher fasting plasma concentration of MGO in obese women with normal glucose tolerance compared to lean individuals, while we did not find a significant difference in the fasting state in men in the current study. This could potentially be explained by the degree of obesity since the average BMI in our previous study was larger than 40 kg/m^2, as compared to a BMI of 30.1 kg/m^2 in our current study. Most importantly, we found a higher iAUC of postprandial dicarbonyls in abdominally obese men, compared to lean men.
Here, we also investigated plasma levels of AGEs, as well as SAF. We showed that abdominally obese men neither had higher fasting plasma levels of AGEs, nor SAF. The only exception was the decreased concentration of CML in abdominally obese men, which is in line with our previous observations, and is likely due to the uptake and accumulation of CML in the expanded adipose tissue of obese individuals, with a consequently lower amount of circulating free levels of CML[4].

Effect of the weight loss intervention

We found that weight loss by caloric restriction reduced fasting plasma MGO and postprandial iAUC of MGO, GO, and 3-DG in abdominally obese individuals. After weight loss, postprandial dicarbonyl stress was reduced to values comparable to those at baseline in the lean group. These data are in line with a reduction in postprandial dicarbonyls in obese women after a 3 weeks caloric restriction[9]. Whilst we previously found the postprandial improvement was largely dependent on decreased fasting levels,

we now show that weight reduction by caloric restriction improves postprandial dicarbonyl iAUCs.

Weight reduction by means of caloric restriction did not reduce free and protein-bound AGEs in plasma, except for protein-bound CEL, which was significantly decreased. CEL is an MGO-derived lysine modification, thus, the decrease in MGO levels by the weight loss intervention could partly explain this finding.
Weight loss did not affect the accumulation of AGEs in skin tissue, as determined by SAF. It is known that modifications of long-lived matrix proteins and cross-links, most notably pentosidine, are generally formed over a long period of time; and that short-term changes in plasma concentrations and AGEs in tissue often do not change in parallel[28]. Indeed, also the plasma concentration of the crosslink pentosidine was not affected by weight loss. Our finding is in accordance with a recent study in morbidly obese individuals where bariatric surgery did not change SAF in the 5 years follow-up period[29].

This study confirmed the effect of weight loss on dicarbonyl stress in a male abdominally obese population. Nonetheless, further research is required to investigate which tissues or cell types contribute most to the increased postprandial dicarbonyl stress in obesity, and to assess the mediating factors by which dicarbonyls were reduced. Multiple mechanisms can contribute to postprandial dicarbonyl stress in obese individuals. Glucose is a major substrate for dicarbonyl compounds[8]. Indeed, the treatment effect was attenuated when correcting for the postprandial iAUC of glucose, and therefore it is likely that glucose is the main substrate for postprandial dicarbonyl formation and that weight loss reduces dicarbonyl stress mainly by improving insulin sensitivity. In other studies, we repeatedly found that higher plasma levels of dicarbonyls, MGO in particular, were associated with incident cardiovascular disease, mortality and kidney disease independently of plasma glucose levels or even HbA1c, in individuals with- and without diabetes[15,16]. This indicates the importance of dicarbonyl compounds as ethiological factors in the development of obesity-related complications. While postprandial glucose levels may largely explain the observed increase in dicarbonyls, lipid peroxidation and the formation of reactive oxygen species[30] could also contribute to the amount of dicarbonyls after a mixed meal. Furthermore, interventions like caloric restriction could influence the detoxification rate of MGO, by induction of GLO1. GLO1 is the key rate-limiting enzyme of the glyoxalase system, converting MGO to D-lactate. Caloric restriction could induce the activation of the stress-responsive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a known inducer of GLO1, by binding to a functional antioxidant-response element in the GLO1 gene[31]. However, we did not find a change in GLO1 expression between lean and obese individual.

Furthermore, there was no change in GLO1 expression by the weight loss intervention. The expression of two other genes known to influence MGO metabolism, AKR1B1 and ALDH2[32], were unchanged as well.
Recently, the ketone body acetoacetate has also been identified as a potential scavenger of MGO[27]. Possibly, higher levels of acetoacetate seen with caloric restriction could scavenge circulating MGO. Acetoacetate and β-hydroxybutyrate are metabolically interconvertible ketone bodies. Under normal conditions, the ratio of β-hydroxybutyrate and acetoacetate is stable around 1:1. During ketosis this ratio is shifted toward higher concentration of β-hydroxybutyrate due to increased fatty acid oxidation[33]. Indeed, after our two-weeks weight stable phase, we found an increase in β-hydroxybutyrate. Hence, we cannot exclude the possibility that ketones after caloric restriction are, at least partly, involved in the decrease of MGO. However, a shift in ketone body and acetoacetate concentrations on their own are unlikely to explain the reduced dicarbonyl stress after weight loss given the available data.

Strengths and limitations

Our study has some limitations. Using strict inclusion criteria, we tried to achieve as little variation in our population as possible. However, selection bias could be a complicating factor for this RCT with participation on a voluntary basis. Furthermore, we only performed measurements at baseline and follow-up. Because of this, we lack information on temporal improvements and improvements taking place during the first weeks of caloric restriction. Although this does not diminish our findings, additional time points could help to elucidate the physiological changes during weight loss. Our study lacks tissue biopsies; we cannot exclude that PBMCs are not fully representative of other tissues with regard to gene expression of GLO1, AKR1B1 and ALDH2. Finally, we only included men, which may reduce the external validity of our results. Given the similarities we found in our previous study with only women included (9), we do not expect that a difference in sex would change the overall conclusion.
The strengths of our study include its RCT design, blinded analyses, intensive monitoring of the participants by a research dietician, very low-dropout rate, use of validated UPLC-MS/MS measurements, and the inclusion of a large panel of AGEs and dicarbonyl compounds. The inclusion of a control group in this trial, which remained weight stable over the course of the intervention, provides a solid baseline when assessing the effects of the intervention. It is also important to note that the dietary program ended with a weight maintenance phase of two weeks after attaining the intended weight loss.

Clinical implications

Our current findings are of potential clinical importance, since dicarbonyl compounds, and MGO in particular, have been identified as potential mediators of cardio-metabolic disease in obesity[34]. We recently demonstrated that higher plasma concentrations of MGO are associated with an accumulation of MGO in adipose tissue[5]. Although the consequences of MGO in adipose tissue are largely unknown, it has been shown that direct incubation of adipocytes with MGO results in increased proliferation indicating that MGO may be involved in the expansion of the adipose tissue. In addition, in an animal model of obesity, elevated MGO levels in serum and adipose tissue were closely associated with the decreased adipose tissue capillarization, adipose blood flow, and the development of insulin resistance[35,36]. In an animal model of obesity, we recently demonstrated that the MGO scavenger pyridoxamine reduced increased pro-inflammatory gene expression levels in visceral adipose tissue, whereas the expression of the anti-inflammatory cytokine adiponectin was increased[5]. Thus, the accumulation of MGO in adipose tissue increases adipose tissue inflammation and may contribute to insulin resistance and the development of type 2 diabetes. Hence, the rapid formation of MGO in the postprandial state could play a role in the association between obesity and consequent metabolic complications. Therefore, it could be valuable to target this early increase in postprandial dicarbonyl stress in obese individuals, for example by actively quenching dicarbonyls or the induction of the glyoxalase system. These treatment strategies are currently under investigation. This is challenging, as compounds investigated so far either lacked efficacy or were linked to potential toxicity[37]. However, compounds that showed potential efficacy, such as the dicarbonyl quenchers pyridoxamine, carnosine or carnosinol, and the co-formulation trans-resveratrol/ hesperetin as inducer of GLO1, have a more favorable pharmacologic profile and are under active investigation[5,38,39].

Conclusion

Postprandial dicarbonyl stress potentially forms an important risk for obesity-related complications. We showed that postprandial dicarbonyl stress is present in obesity, and can be reversed by a dietary weight loss intervention. Future work should investigate whether targeting postprandial dicarbonyl stress in obesity can prevent obesity-related complications, type 2 diabetes and cardiovascular disease.

Acknowledgements

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nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussions.

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Supplemental materials

Table S2.1 Gene expression analysis of GLO1, ALDH2, and AKR1B1.

	Lean vs. Obese (n=15 vs. 29)		Weight loss (n=14)		Weight stable (n=15)	
	Difference	P value	Change	P value	Change	P value
GLO1	-1.05	0.34	1.04	0.16	1.01	0.65
ALDH2	1.15	0.11	1.00	0.93	-1.02	0.70
AKR1B1	1.09	0.10	-1.01	0.70	1.01	0.79

Data are changes in gene expression: lean vs. obese, weight loss follow-up vs. baseline, weight stable follow-up vs. baseline. Differences between groups were assessed by means of an independent samples t-test, with empirical Bayes correction. GLO1: glyoxalase 1, AKR1B1: aldo-keto reductase family 1 member B, ALDH2: aldehyde dehydrogenase.

Table S2.2 Cross-sectional analysis of ketone bodies and the effect of weight loss on ketone body concentrations.

	Cross-sectional analysis			Weight loss (n=13)		Weight stable (n=12)		Treatment effect	
	Lean (n=14)	Obese (n=25)	P value[†]	BL	FU	BL	FU	Median of change[‡]	P value[§]
Acetoacetate (µmol/L)	34.8 [27.2-50.3]	32.6 [22.3-42.3]	0.343	33.8 [23.1-52.1]	39.7 [34.7-75.4]	29.8 [20.4-41.7]	28.8 [20.5-46.4]	5.2 vs. -0.9	0.150
B-Hydroxybutyrate (µmol/L)	52.7 [30.9-114.1]	46.5 [33.4-77.1]	0.444	52.6 [34.1-78.3]	82.1 [35.1-302.8]	46.5 [32.8-77.7]	51.4 [34.9-62.1]	20.8 vs. -8.0	0.044*

Data are presented as median [IQR]. [(†)]Baseline differences between obese and lean men were assessed by means of an independent samples t-test. [(‡)]The medians of the changes (delta intervention) in respectively the weight loss group and the control (weight stable) group are given. [(§)]Differences in changes between the weight loss treatment and weight stable control treatment were tested using one-way ANCOVA with adjustment for baseline values (*p<0.05). Variables were Log transformed prior to analysis.

Table S2.3 Cross-sectional analysis of postprandial dicarbonyls.

	Lean (n=25)	Obese (n=52)	P value
Postprandial concentrations			
MGO iAUC (nmol/L x min)	3588 ± 7652	9288 ± 9070	0.008**
GO iAUC (nmol/L x min)	7406 ± 19940	17152 ± 21339	0.059
3-DG iAUC (nmol/L x min)	21532 ± 28806	44511 ± 33144	0.004**
Glucose iAUC (mmol/L x min)	137 ± 157	225 ± 191	0.049*

Data are presented as mean ± SD. Baseline differences between obese and lean men were assessed by means of an independent samples t-test (*p<0.05, **p<0.01). Dicarbonyls: MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone. iAUC: incremental area under the curve

Table S2.4 Effect of weight loss on postprandial dicarbonyls.

	Weight loss (n=23)		Weight stable (n=26)		Treatment effect	
	Baseline	Follow-up	Baseline	Follow-up	Mean change (95% CI)[†]	P value[‡]
Postprandial concentrations						
MGO iAUC (nmol/L x min)	9743 ± 9324	2426 ± 10900	8916 ± 9532	7583 ± 7166	-5280 (-10526, -33)	0.049*
GO iAUC (nmol/L x min)	14997 ± 21121	7551 ± 19255	18968 ± 22867	19567 ± 18608	-11329 (-22162, -495)	0.041*
3-DG iAUC (nmol/L x min)	40145 ± 32016	13020 ± 27309	49257 ± 34849	38301 ± 34686	-20175 (-35000, -5351)	0.009**
Glucose iAUC (mmol/L x min)	183 ± 159	86 ± 158	268 ± 211	228 ± 209	-83 (-164, -2)	0.044*

Data are presented as mean ± SD. Differences in changes between the weight loss treatment and weight stable control treatment, were tested using one-way ANCOVA with adjustment for baseline values. [(†)]Adjusted mean changes (estimated marginal means) with 95% CI are given. [(‡)]All P values are obtained from the ANCOVA analysis with adjustment for baseline values (*p<0.05, **p<0.01). Dicarbonyls: MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone. iAUC: incremental area under the curve.

Table S2.5 Treatment effect on postprandial dicarbonyl iAUC's without and with adjustment for glucose.

	Treatment effect[†]		Glucose-adjusted treatment effect[‡]	
	Mean change (95% CI)	P value	Mean change (95% CI)	P value
MGO iAUC (nmol/L x min)	-5280 (-10526, -33)	0.049	-2913 (-8416, 2589)	0.292
GO iAUC (nmol/L x min)	-11329 (-22162, -495)	0.041	-9213 (-20838, 2411)	0.117
3-DG iAUC (nmol/L x min)	-20175 (-35000, -5351)	0.009	-2255 (-7111, 2601)	0.354

[†] Differences in changes between the weight loss treatment and weight stable control treatment were tested using one-way ANCOVA with adjustment for baseline values. [‡] Additional adjustment was performed for baseline and follow-up glucose iAUC levels. iAUC: incremental area under the curve.
Dicarbonyls: MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone.

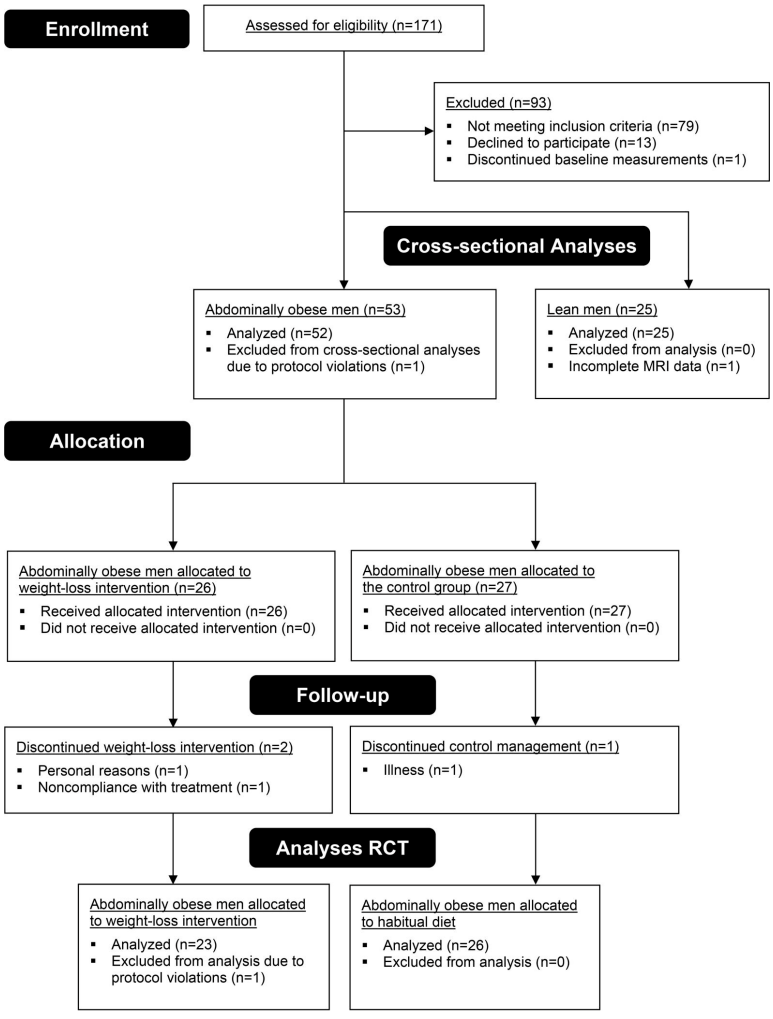

Figure S2.1 Flow diagram of participants.
After screening, 25 lean men were eligible for participation, and all completed the study. Baseline measurements were performed in 54 abdominally obese men; one dropped out for personal reasons before completing the measurements. For the cross-sectional analyses, all lean men and 52 abdominally obese men were included; one was excluded from analyses due to protocol violations (use of anti-hypertensive medication during participation). One lean individual did not complete the MR imaging due to claustrophobia. A total of 53 abdominally obese men were randomized to weight loss intervention or weight-stable control groups. One individual discontinued control treatment because of illness; one dropped out because of noncompliance with the program to lose weight; and another individual dropped out for personal reasons after assignment to the weight loss treatment. One individual completed the trial but was excluded from all analyses because of protocol violations. In agreement with the protocol, the study was stopped after 50 follow-up measurements were completed. Few harms were observed during this study; one individual had an incidental finding on MRI that required further analysis, and another individual developed thrombophlebitis after the follow-up measurements.

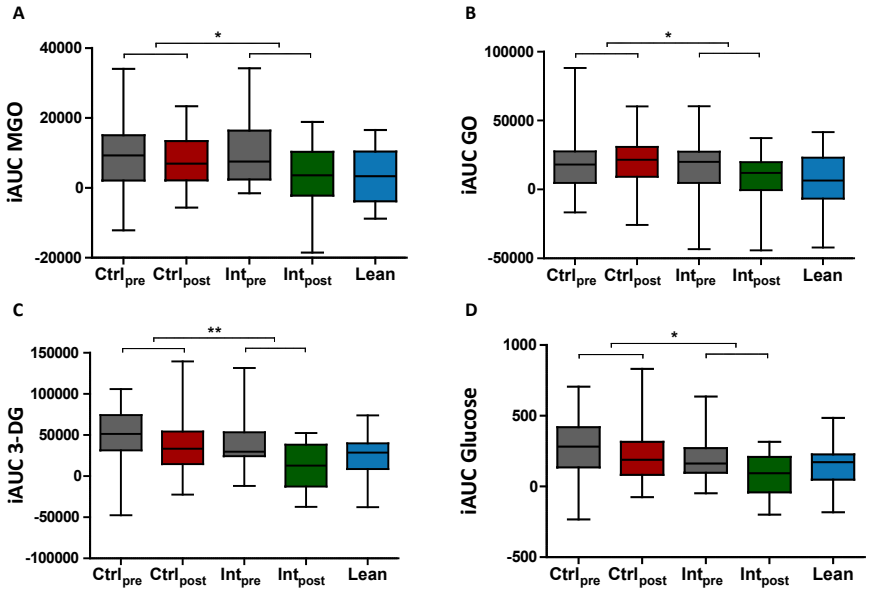

Figure S2.2 Effect of the weight loss intervention in comparison to lean individuals.
Graph (A) shows MGO iAUC in nmol/L x min. (B-C) show the same for GO and 3-DG. (D) shows glucose iAUC in mmol/L x min. From left to right: control at baseline, control at follow-up (red), weight loss at baseline, weight loss at follow-up (green), and lean individuals from the cross-sectional analysis (blue). Boxplots indicate median, 25th and 75th percentile, and min to max whiskers. Differences in changes between the weight loss intervention and weight stable control were tested using one-way ANCOVA with adjustment for baseline values (*p≤0.05, **p≤0.01). iAUC: incremental area under the curve, MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone.

Chapter 3

Physical activity and markers of glycation in older individuals: data from a combined cross-sectional and randomized controlled trial (EXAMIN AGE)

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Abstract

Background

Advanced glycation endproducts (AGEs) are protein modifications that are predominantly formed from dicarbonyl compounds that arise from glucose and lipid metabolism. AGEs and sedentary behavior have been identified as a driver of accelerated (vascular) aging. The effect of physical activity on AGE accumulation is unknown. Therefore, we investigated whether plasma AGEs and dicarbonyl levels are different across older individuals that were active or sedentary and whether plasma AGEs are affected by high intensity interval training (HIIT).

Methods

We included healthy older active (HA, *n*=38, 44.7% female, 60.1±7.7 years old) and healthy older sedentary (HS, *n*=36, 72.2% female, 60.0±7.3 years old) individuals as well as older sedentary individuals with increased cardiovascular risk (SR, *n*=84, 50% female, 58.7±6.6 years old). The SR group was randomized into a 12-week walking-based HIIT program or control group. We measured protein-bound and free plasma AGEs and dicarbonyls by UPLC-MS/MS at baseline and after the HIIT intervention.

Results

Protein-bound AGE N^{ε}-(carboxymethyl)lysine was lower in SR (2.6±0.5 µmol/L) and HS (3.1±0.5 µmol/L) than in HA (3.6±0.6 µmol/L; $p<0.05$) and remained significantly lower after adjustment for several potential confounders. None of the other glycation markers were different between HS and HA. HIIT did not change plasma AGEs and dicarbonyls in SR.

Discussion

Although lifestyle interventions may act as important modulators of cardiovascular risk, HIIT is not a potent short-term intervention to reduce glycation in older individuals, underlining the need for other approaches, such as pharmacological agents, to reduce AGEs and lower cardiovascular risk in this population.

Introduction

Advanced glycation endproducts (AGEs) are linked to a plethora of age-related conditions including cardiovascular disease (CVD)[1-4] and vascular diseases associated with diabetes[5,6]. As such, this heterogeneous family of sugar-protein modifications has been identified as a potential driver of the aging process.[6] The formation of AGEs is complex, but recent data have demonstrated that glycolysis- and lipid-oxidation-derived dicarbonyl compounds such as methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG) are important precursors in the rapid formation of AGEs such as N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) and 5-hydro-5-methylimidazolone (MG-H1)[7]. These toxic dicarbonyls and their derived AGEs are thought to be central players in the development of age-related diseases like diabetes mellitus and CVD[8-10]. Therefore, strategies to lower the burden of high levels of AGEs may improve cardiovascular health. However, clinical studies targeting the formation of dicarbonyls and AGEs with specific inhibitors did not achieve clinical implementation due to safety concerns and/or limited potency to effectively lower AGEs in-vivo[11].

Lifestyle intervention can be employed to effectively lower the burden of glycation in humans[12]. Physical activity has a profound impact on insulin sensitivity and the rate of glycolysis and lipid oxidation[13]. Furthermore, physical exercise has been shown to attenuate the age-related decline of cardiovascular function[14-16] and reduces both cardiovascular morbidity and mortality[17,18]. These positive effects of physical exercise are believed to be largely due to an improvement of metabolic control, and may therefore be in part due to a reduction of AGEs and dicarbonyls. Indeed, animal studies have suggested that physical exercise may reduce dicarbonyl stress and AGEs[19]. However, human studies linking exercise training with dicarbonyl stress or AGEs are sparse and have yielded conflicting outcomes with either positive[20-22], negative[23], or no effects[24,25] on AGE levels, presumably due to heterogeneities between study populations and techniques used to measure AGEs. In the current study, we tested the hypothesis that sedentary behavior is associated with increased levels of glycation and that an exercise intervention reduces the rate of glycation. Specifically, we investigated whether AGE and dicarbonyl levels differed between older active and sedentary individuals with and without cardiovascular risk factors. Additionally, we assessed the effect of high intensity interval training in a randomized controlled trial in older sedentary individuals at increased cardiovascular risk.

Methods

Study design and procedures

The Exercise, Arterial Crosstalk Modulation, and Inflammation in an Aging population (EXAMIN AGE) study was designed to investigate the influence of physical activity on healthy aging and the effects of high intensity training as described in detail recently[16,26,27]. In the cross-sectional part, we examined the association between physical activity and fitness, and plasma AGEs and dicarbonyls in healthy older active (HA, *n*=38) and healthy sedentary (HS, *n*=36) individuals, as well as older sedentary individuals with increased cardiovascular risk (SR, *n*=84). For the interventional part, the SR group was randomized into a 12-week walking-based high-intensity interval training (HIIT) group (n=44) or a control group with standard physical activity recommendations based on current guidelines (n=40)[28], in order to examine the effects of HIIT on AGE and dicarbonyl levels in older adults (see flow-chart, Supplementary Figure S3.1). All procedures were performed as previously described[26].

Briefly, HA and HS as well as SR participants were recruited and enrolled in the cross-sectional study based on data from the first visit. Recruitment of the SR group was based on the agreement to take part in the exercise program following the cross-sectional assessment (which served as a baseline examination for the consecutive intervention study). At the first visit, anthropometric measurements were performed, physical activity was assessed, and 24h blood pressure monitoring and blood sampling were performed. On a separate visit cardiopulmonary exercise testing was performed to assess maximal oxygen uptake (VO_2max) as an estimate of peak endurance performance. For the intervention study, all measurements were repeated during the follow-up visit 12 weeks later, n=40 of the HIIT group and n=34 of the control group completed the intervention study.

VO_2max was measured using the Cortex Metalyzer R 3B metabolic test system (Cortex Biophysik GmbH, Leipzig, Germany). All participants wore an Aipermotion 440 accelerometer (Aipermon GmbH, Munich, Germany) for six consecutive days on their left hip. From the five most active days, we calculated walking and fast walking in minutes per day, distance in meter per day and total steps per day using the AiperView 440 and ActiCoach MPAT2Viewer Software (Aipermon GmbH, Munich, Germany). The Freiburg Questionnaire of Physical Activity (FQPA) was used to assess self-reported sport activities in metabolic equivalents (METs) per week based on the Ainsworth Compendium[29].

All participants were medically examined by the study physician before inclusion, and written informed consent was obtained from all eligible individuals. All study procedures and ethical considerations have been previously described[26]; this study was conducted in

accordance with the Declaration of Helsinki and approved by the Ethics Committee of Northwestern and Central Switzerland (EKNZ-2015-351). The EXAMIN AGE study was registered on ClinicalTrials.gov (NCT02796976) in June 2016.

Study population and in-/exclusion criteria

Both the healthy active and healthy sedentary group included healthy men and women aged 50-80 years without cardiovascular risk factors, with an active lifestyle (>9 MET/week) or a sedentary lifestyle (≤3 MET/week) respectively. Individuals with a history of cardiovascular, pulmonary or chronic inflammatory disease, a blood pressure ≥140/90 mmHg during 24h monitoring, macular degeneration or glaucoma, a history of smoking or any additional risk factors, were excluded.
The SR group was aged 50-80 years, and had at least two additional cardiovascular risk factors (being either obesity, elevated triglyceride or LDL levels, decreased HDL levels, elevated blood pressure, elevated plasma glucose, or current smoking; the risk factor distribution is shown in Supplementary Table S1).[26] Individuals with decompensated cardiovascular, pulmonary or chronic inflammatory disease, macular degeneration, glaucoma, or compromising orthopedic problems were excluded from the study.

Exercise intervention

The SR group were randomized to the intervention or control group by an independent research assistant. The physical exercise intervention comprised of a 12-week supervised Nordic Walking-based HIIT, performed three times per week. In the first week, the participants trained with an intensity of 75% of their maximum heart rate to get familiarized with a continuous walking-based training. In the second week, a stepwise increase of the intensity up to 80-90% of their maximum heart rate was performed. In the following 10 weeks, the participants performed the HIIT based on the following protocol with a total duration of 45 minutes per session: warm-up for 10 minutes at 60-70% of maximum heart rate, followed by a high-intensity interval consisting of 4x4 minutes at 80-90% with 3minutes of active recovery at 60-70%, and a cool-down of 10 minutes at 60-70%. Heart rate was monitored during training by standard heart rate sensors. The control group received physical activity recommendations based on the European Guidelines on Cardiovascular Disease Prevention in Clinical Practice[28].

Measurement of plasma dicarbonyls and AGEs

All plasma samples were stored at -80^0 Celsius prior to analyses. Plasma levels of dicarbonyls and AGEs were measured in EDTA plasma samples. Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to determine

plasma levels of the free and protein-bound AGEs CML, CEL MG-H1[30], and the dicarbonyls MGO, GO, and 3-DG[31]. Coefficients of variation were all below 10%. To infer influence of plasma protein content on protein-bound AGE levels we measured total plasma protein with the Bradford reaction.

Statistics

Baseline characteristics were presented as mean ± standard deviation or as interquartile ranges when applicable. Differences at baseline between the HA, HS and SR groups were tested with one-way ANOVA. We used linear regression to assess associations between physical activity classification (determinant) and plasma AGE levels or plasma dicarbonyls (outcome). Betas were expressed as standardized regression coefficients. We adjusted for potential confounders: Model 1: crude analysis, Model 2: crude analysis + confounders (age, sex, fat and muscle mass, LDL- and glucose levels). The associations with protein-bound AGEs were also adjusted for total plasma protein levels. Next, we assessed the effect of the HIIT intervention on the plasma glycation markers, using a one-way ANCOVA analysis with correction for baseline values. We Ln-transformed protein-bound MG-H1 and the free AGEs to achieve a normal distribution of the residuals in these analyses.

Results

Table 3.1 shows the baseline characteristics of the cross-sectional EXAMIN AGE study comparing HA, HS and SR groups.

Table 3.1 Participants' characteristics.

	HA (n=38)	HS (n=36)	SR (n=84)
Sex (Female, %)	44.7	72.2	50.0
Age (years)	60.1 ± 7.7	60.0 ± 7.3	58.7 ± 6.6
BMI (kg/m^2)	22.1 ± 1.7	24.8 ± 2.4	33.2 ± 4.1
Fat mass (kg)	13.0 ± 3.8	22.8 ± 5.9	37.9 ± 9.7
Muscle mass (kg)	28.6 ± 4.3	25.9 ± 4.8	31.6 ± 6.9
Current smoker (%)	0	0	33.0
Systolic BP (mm Hg)	127.8 ± 15.3	127.2 ± 14.9	132.3 ± 14.3
Diastolic BP (mm Hg)	77.8 ± 8.1	81.0 ± 8.2	87.3 ± 9.7
Fasting glucose (mmol/L)	4.7 ± 0.4	4.7 ± 0.5	5.8 ± 1.8
LDL cholesterol (mmol/L)	2.9 ± 0.7	3.1 ± 0.8	3.2 ± 0.8
HDL cholesterol (mmol/L)	2.0 ± 0.4	1.7 ± 0.4	1.3 ± 0.3
Triglycerides (mmol/L)	1.0 (0.7-1.1)	1.0 (0.8-1.3)	1.6 (1.1-1.8)
VO_2 max (ml/min/kg)	42.5 ± 8.3	29.9 ± 4.3	26.0 ± 4.3
Accelerometer			
Walking (min/day)	108 ± 45	104 ± 39	91 ± 39
Fast walking (min/day)	29 (23-41)	16 (6-28)	11 (4-20)
Distance (meter/day)	9310 ± 3385	6424 ± 2696	5568 ± 2397
Steps (n)	13267 ± 4870	10105 ± 3828	8712 ± 3588
Glycation markers			
Protein-bound CML (nmol/L)	3586 ± 606.5	3091 ± 466.1	2556 ± 504.0
Protein-bound CEL(nmol/L)	2254 ± 467.2	2315 ± 592.2	2493 ± 642.1
Protein-bound MG-H1 (nmol/L)	1762 (1572-2324)	1535 (1294-2085)	1325 (1112-1565)
Free CML (nmol/L)	90.0 (75-110.5)	93.5 (70.3-115.3)	99.0 (80.5-141.0)
Free CEL (nmol/L)	49.0 (38.5-55.0)	49.0 (36.3-57.0)	54.5 (44.0-64.0)
Free MG-H1 (nmol/L)	100.0 (65.5-155.0)	109.5 (73.8-130)	100.5 (77.0-157.3)
Methylglyoxal (nmol/L)	757.9 ± 292.6	652.1 ± 249.7	665.0 ± 267.8
Glyoxal (nmol/L)	1372 ± 343.5	1163 ± 354.7	1195 ± 345.9
3-Deoxyglucosone (nmol/L)	1143 ± 101.7	1121 ± 154.0	1412 ± 455.6

Healthy older active subjects (HA), healthy older sedentary subjects (HS) and older sedentary subjects at risk (SR). Blood pressure (BP), low density lipoproteins (LDL), high density lipoproteins (HDL). Glycation markers: protein-bound and free N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), 5-hydro-5-methylimidazolone (MG-H1), and the dicarbonyl compounds methylglyoxal, glyoxal, and 3-deoxyglucosone. Represented data are mean ± SD or median and interquartile ranges, as appropriate.

Plasma glycation levels in older active and sedentary subjects

To investigate the influence of active and sedentary behavior on plasma levels of glycation, we first compared crude plasma AGE and dicarbonyl levels across the HA, HS, SR groups. We found that plasma protein-bound CML levels were significantly lower in HS compared to HA, and still lower in SR (Table 3.1, Figure 3.1). GO, a major dicarbonyl precursor for CML, was also significantly lower in HS and SR. We made similar observations for MGO and MGO-derived MG-H1, although this was significant only for MG-H1 in the SR group (Figure 3.1). In line with plasma glucose levels (Table 3.1), levels of the glucose-derived dicarbonyl 3-DG were significantly higher in SR, but not in HS.

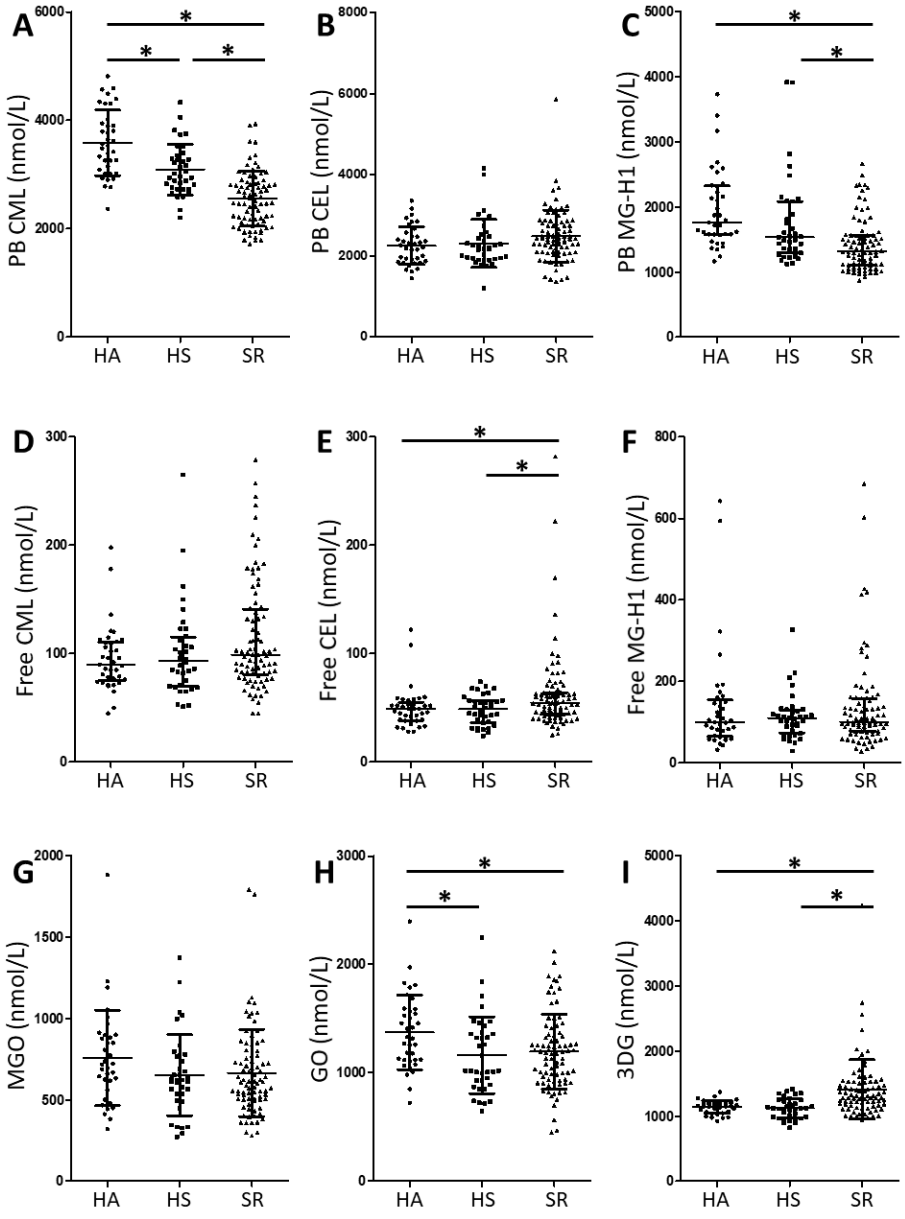

Figure 3.1 Plasma levels of AGEs and dicarbonyl compounds in healthy older active subjects (HA, n=38), healthy older sedentary subjects (HS, n=36) and older sedentary subjects at risk (SR, n=84). Using UPLC-MS/MS we measured levels of protein-bound CML (A), CEL (B), MG-H1 (C), free CML (D), CEL (E) and MG-H1 (F) and the dicarbonyl compounds MGO (G), GO (H) and 3DG (I). Represented data are mean ± SD (A, B, G-I) or median and interquartile ranges (C-F), as appropriate. Differences were compared with ANOVA with Bonferroni correction. * $p<0.05$. Skewed variables were Ln-transformed

Next, we performed linear regression analyses to address potential confounding with regard to the associations between the sedentary groups and plasma AGE and dicarbonyl levels. Adjustment for potential confounders (sex, age, fat and muscle mass, LDL- and glucose levels) strongly attenuated the association between lower CML levels and the HS and SR groups relative to HA (Table 3.2, Model 2). The point estimates of the association between lower plasma MG-H1 levels and SR, relative to HA, were slightly attenuated after adjustment for potential confounders (Table 3.2, Model 1-2). Adjustment for total plasma protein did not influence any of the associations with the protein-bound AGEs (data not shown).
The association between lower GO levels and SR was attenuated and lost statistical significance after adjustment for potential confounders (Table 3.3, Model 2), while the association with HS remained largely unaffected. The association between SR and higher 3DG levels reversed when we adjusted for potential confounders (Table 3.3, Model 2).

Associations between plasma glycation levels and VO_2 max

Next, we pooled all subjects (HA and HS and SR groups) to study the associations between physical fitness and plasma AGEs and dicarbonyls. In line with the lower plasma CML levels in HS and SR, we found that a higher VO_2 max, as a marker of physical fitness, was associated with higher plasma CML levels (Table 3.3, Model 1). This association was attenuated after adjustment for potential confounders (sex, age and fat and muscle mass, LDL- and glucose levels (Table 3.3, Model 2). Although a higher VO_2 max was also associated with MG-H1 levels in a crude analysis, this association lost statistical significance after adjustment for potential confounders (Table 3.3, Model 1-2). Likewise, the association between a higher VO_2 max and lower 3DG levels was no longer significant after adjustment for potential confounders (Table 3.3, Model 1-2).

Table 3.2 Associations between plasma AGE and dicarbonyl levels of healthy older sedentary subjects (HS) and older sedentary subjects at risk (SR) compared to healthy older active subjects (HA), adjusted for potential confounders.

Model	HS	SR	HS	SR	HS	SR
	PB CML		PB CEL		Ln-PB MG-H1	
1	**-0.74 (-1.09 to -0.38)**	**-1.51 (-1.82 to -1.21)**	0.10 (-0.36 to 0.56)	**0.40 (0.01 to 0.79)**	-0.27 (-0.69 to 0.16)	**-0.96 (-1.32 to -0.60)**
2	**-0.55 (-0.93 to -0.16)**	**-0.87 (-1.39 to -0.35)**	0.17 (-0.33 to 0.66)	0.23 (-0.44 to 0.90)	-0.22 (-0.68 to 0.25)	-0.93 (-1.56 to 0.30)
	Ln-Free CML		Ln-Free CEL		Ln-Free Ln-MG-H1	
1	0.09 (-0.36 to 0.54)	0.33 (-0.06 to 0.71)	-0.07 (-0.49 to 0.36)	**0.38 (0.02 to 0.74)**	-0.11 (-0.56 to 0.34)	-0.05 (-0.42 to 0.34)
2	0.16 (-0.32 to 0.63)	0.34 (-0.29 to 0.98)	-0.13 (-0.54 to 0.27)	**-0.04 (0.58 to 0.51)**	-0.11 (-0.56 to 0.34)	-0.13 (-0.73 to 0.47)
	MGO		GO		3DG	
1	-0.39 (-0.86 to 0.08)	-0.36 (-0.76 to 0.03)	**-0.59 (-1.04 to -0.14)**	**-0.52 (-0.91 to -0.14)**	-0.06 (-0.39 to 0.27)	**0.64 (0.36 to 0.92)**
2	-0.44 (-0.94 to 0.07)	-0.40 (-1.08 to 0.28)	**-0.51 (-1.01 to -0.02)**	-0.28 (-0.94 to 0.39)	**-0.17 (-0.34 to 0.00)**	-0.12 (-0.34 to 0.11)

Betas are expressed per standard deviation plasma AGE, HS (n=36) and SR (n=84) versus HA (n=38) as the reference group. The free AGEs and protein-bound MG-H1 were Ln transformed prior to analyses.

Model 1: crude

Model 2: 1+ age, sex, fat mass and muscle mass, fasting LDL and glucose levels

Table 3.3 Associations between VO_2 max and plasma AGEs and dicarbonyl levels.

Model	PB CML	PB CEL	Ln-PB MG-H1
1	**0.48 (0.34 to 0.62)**	-0.06 (-0.23 to 0.10)	**0.29 (0.14 to 0.44)**
2	**0.27 (0.05 to 0.49)**	-0.07 (-0.35 to 0.22)	0.17 (-0.10 to 0.44)
Model	**Ln-Free CML**	**Ln-Free CEL**	**Ln-Free MG-H1**
1	-0.08 (-0.23 to 0.07)	-0.07 (-0.21 to 0.07)	0.10 (-0.05 to 0.25)
2	-0.14 (-0.40 to 0.12)	0.01 (-0.22 to 0.23)	0.06 (-0.20 to 0.31)
Model	**MGO**	**GO**	**3DG**
1	0.08 (-0.08 to 0.24)	0.13 (-0.03 to 0.29)	**-0.21 (-0.33 to -0.09)**
2	0.09 (-0.20 to 0.38)	0.11 (-0.18 to 0.39)	0.03 (-0.07 to 0.13)

Pooled analysis of all subjects from the HA (n=38), HS (n=36) and SR (n=84) group combined. Glycation markers: protein-bound (PB) and free N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), 5-hydro-5-methylimidazolone (MG-H1), and the dicarbonyl compounds methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3DG). Beta expressed as standard deviation plasma AGE per standard deviation VO_2 max. The free AGEs and protein-bound MG-H1 were Ln transformed prior to analyses.
Model 1: crude.
Model 2: 1+ age, sex, fat mass and muscle mass, fasting LDL and glucose levels.

A 12-week high-intensity interval training and plasma glycation markers in sedentary individuals at increased cardiovascular risk

Next, we studied whether HIIT can reduce plasma AGEs and dicarbonyls. Although the intervention significantly increased VO_2 max and muscle mass and decreased weight, fat mass, BMI, and LDL after 12 weeks (Table 3.4)[16], we found no statistically significant effects on plasma protein-bound or free CML, CEL and MG-H1 levels or plasma MGO, GO and 3DG levels by the HIIT intervention (Figure 3.2). There were no significant mean differences between control and HIIT for any of the glycation markers when we adjusted for the base-line values (Table 3.5). Additionally, we analyzed the trial excluding individuals using glucose-lowering medication (n=6 in the control and n=6 in the HIIT group), this did not alter any of the results (data not shown).

Table 3.4 Participants` characteristics of older sedentary individuals with increased cardiovascular risk (SR) before and after intervention.

	Control (n=34)		Intervention (n=40)	
	Baseline	**Follow-up**	**Baseline**	**Follow-up**
BMI	32.9 ± 4.8	32.5 ± 4.7	32.9 ± 3.3	32.5 ± 3.4
Current smoker (%)	32.4	35.4	30.0	25.0
Systolic BP (mm Hg)	128.7 ± 13.5	134.2 ± 14.3	134.2 ± 14.3	133.8 ± 11.9
Diastolic BP (mm Hg)	85.4 ± 10.3	88.2 ± 9.5	88.2 ± 9.5	86.9 ± 7.1
Fasting glucose (mmol/L)	5.8 ± 1.4	5.6 ± 1.2	5.8 ± 2.1	5.7 ± 1.7
LDL cholesterol (mmol/L)	3.0 ± 0.7	2.9 ± 0.8	3.3 ± 0.8	3.0 ± 0.8
HDL cholesterol (mmol/L)	1.4 ± 0.3	1.4 ± 0.4	1.3 ± 0.3	1.3 ± 0.3
Triglycerides (mmol/L)	1.6 (1.1-1.8)	1.5 (1.1-2.3)	1.5 (1.2-1.8)	1.4 (1.2-1.70)
VO_2 max (ml/min/kg)	26.1 ± 5.0	25.0 ± 4.0	26.4 ± 3.8	28.7 ± 4.1*
Accelerometer				
Walking (min/day)	97 ± 39	94 ± 42	89 ± 41	92 ± 39
Fast walking (min/day)	10 (5-20)	8 (3-20)	12 (4-21)	14 (7-24)
Distance (meters/day)	5868 ± 2495	5615 ± 2652	5556 ± 2428	5796 ± 2207
Steps (n)	9256 ± 3648	8920 ± 4108	8591 ± 3628	9065 ± 3497
Glycation markers				
Protein-bound CML (nmol/L)	2647.5 ± 547.1	2618.6 ± 607.8	2587.2 ± 457.0	2602.8 ± 429.4
Protein-bound CEL (nmol/L)	2435.5 ± 515.9	2220.9 ± 540.5	2471.0 ± 521.3	2446.5 ± 561.5
Protein-bound MG-H1 (nmol/L)	1273 (1056-1499)	1435 (1212-1663)	1481 (1121-1618)	1337 (1202-1536)
Free CML (nmol/L)	95.5 (76.8-149.3)	103.9 (78.2-142.4)	102.0 (83.0-132.0)	105.2 (82.5-126.7)
Free CEL (nmol/L)	57.0 (41.0-71.8)	54.7 (38.6-67.8)	54.0 (44.0-61.8)	57.0 (43.2-78.7)
Free MG-H1 (nmol/L)	104.0 (62.75-138.5)	120.6 (85.0-186.7)	100.0 (77.0-159.0)	126.1 (82.0-180.9)
Methylglyoxal (nmol/L)	643.1 ± 276.0	830.3 ± 346.4	684.7 ± 203.1	1013.8 ± 627.9
Glyoxal (nmol/L)	1218.9 ± 335.9	1411.5 ± 453.7	1227.6 ± 362.0	1439.2 ± 437.1
3-Deoxyglucosone (nmol/L)	1368.1 ± 301.4	1368.5 ± 273.8	1455.5 ± 550.8	1378.2 ± 454.3

Blood pressure (BP), low density lipoproteins (LDL), high density lipoproteins (HDL). Data are represented as mean ± standard deviation or as interquartile ranges, as appropriate. *P<0.05, versus control differences were compared across group allocation at follow-up with ANCOVA adjusting for baseline values. Glycation markers: protein-bound and free N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), 5-hydro-5-methylimidazolone (MG-H1), and the dicarbonyl compounds methylglyoxal (MGO), gyoxal (GO), and 3-deoxyglucosone (3DG).

Table 3.5 Mean differences between control and HITT for plasma AGEs and dicarbonyls.

Glycation markers (nmol/L)	% change control (ratio geom. means FU-BL values)	% change intervention (ratio geom. means FU-BL values)	Mean difference (control - intervention)	Lower bound 95% CI	Upper bound 95% CI
Protein-bound CML	-1.5	-0.8	-20.17	-167.71	127.37
Protein-bound CEL	-9.3	-1.9	-213.48	-459.97	33.01
Protein-bound MG-H1	11.1	-4.3	0.082	-0.028	0.19
Free CML	2.6	-2.4	0.040	-0.103	0.183
Free CEL	-6.5	6.8	-0.097	-0.242	0.047
Free MG-H1	9.6	10.5	0.013	-0.192	0.218
MGO	24.4	34.1	-161.70	-398.12	74.72
GO	10.3	17.0	-25.45	-229.42	178.53
3-DG	0.5	-2.1	54.51	-33.33	142.34

Mean differences and % change of plasma AGEs and dycarbonyls, between the high intensity interval training (HIIT) intervention (n=40) and control group (n=34) in older sedentary individuals at risk (SR). In individuals randomized to either control or HIIT intervention, we measured at baseline and follow-up levels of protein-bound CML, CEL, MG-H1, free CML, free CEL, free MG-H1, and the dicarbonyl compounds MGO, GO and 3DG with UPLC-MS/MS. Differences were compared across group allocation at follow-up with ANCOVA adjusting for baseline values. Represented data are mean differences between the control and intervention group with 95% confidence intervals. Protein-bound MG-H1 and free AGEs were ln transformed to achieve a normal distribution of the residuals and therefore presented on the Ln scale.

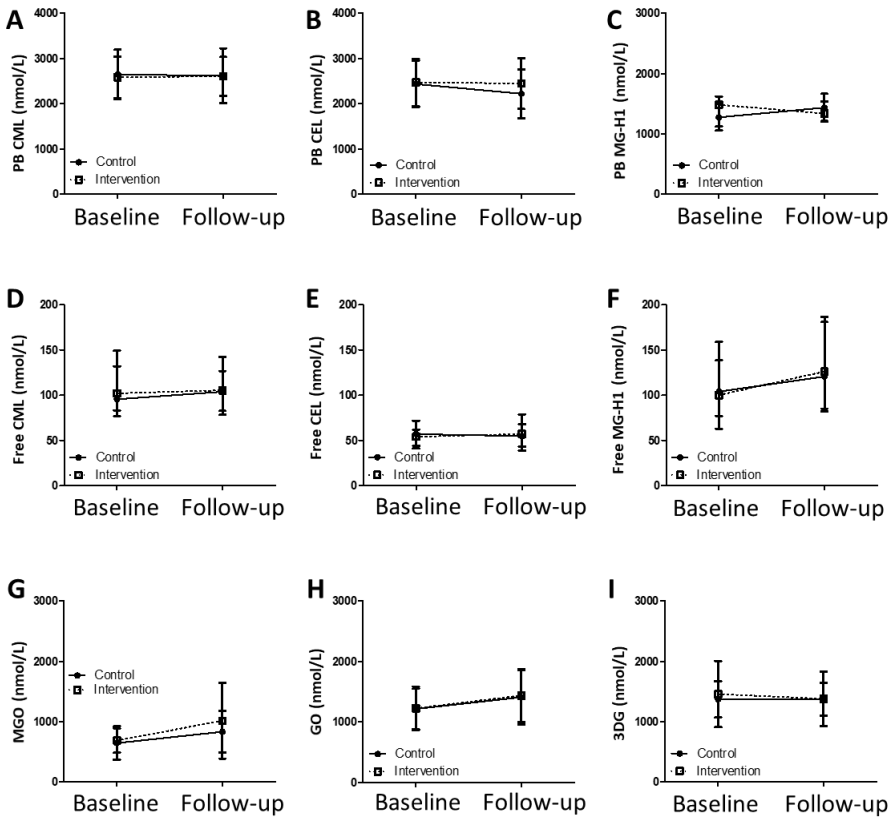

Figure 3.2 Effect of a high intensity interval training (HIIT) on plasma AGE and dycarbonyl compounds in older sedentary individuals at risk (SR). In individuals randomized to either control (n=34) or HIIT (n=40) intervention, we measured at baseline and follow-up levels of protein-bound CML (A), CEL (B), MG-H1 (C), and free CML (D), CEL (E) and MG-H1 (F) and the dicarbonyl compounds MGO (G), GO (H) and 3DG (I) with UPLC-MS/MS. Represented data are mean ± SD (A,B, G-I) or median and interquartile ranges (C-F), as appropriate. Differences were compared across group allocation at follow-up with ANCOVA adjusting for baseline values.

Discussion

The main finding from the cross-sectional part of this study is that plasma levels of glycation were not higher in sedentary subjects (either the HS or SR groups) as compared with active subjects (the HA group). Protein-bound plasma levels of CML in fact appeared to be lower in the HS and SR groups. In line, higher CML levels were associated with a higher VO_2 max, but no other consistent associations were found between markers of glycation and sedentary behavior or VO_2 max. A second main finding

is that a 12-week HIIT intervention did not influence any of the plasma glycation markers.

Our current cross-sectional findings are in line with our previous study showing higher CML concentrations in lifelong endurance athletes compared to sedentary individuals[23]. We also confirm a positive association between CML and VO_2 max. Although we do not have a clear explanation for the finding of higher plasma CML levels in more active individuals, exercise generally promotes tissue repair, turnover of matrix proteins and the breakdown of cross-links in the vessel wall[7,32,33], which may lead to higher levels of circulating protein-bound AGEs. Another explanation for the higher AGEs levels in HA individuals could be a higher metabolic rate, resulting in an increased formation of AGEs[34,35]. Furthermore, greater physical activity is associated with a higher caloric intake and we recently demonstrated that higher energy intake (kcal/day) is associated with a higher intake of dietary AGEs[36]. Therefore, higher energy intake in active individuals may, at least in part, explain the increased levels of CML in these individuals. Unfortunately, we could not take potential confounding by dietary factors fully into account in the current study.

So far, human studies linking exercise training with plasma AGE levels have yielded conflicting results. It has been demonstrated that a 12-month Tai Chi intervention reduced plasma AGEs in healthy middle-aged adults[20] and that physical training in patients with either HIV[21], or breast cancer[22] also reduced circulating glycation levels. In agreement with these studies, an animal study showed a reduction in plasma dicarbonyls and CML in rats subjected to physical exercise (treadmill running)[19], although the interventions and metabolic effects in animals are, in general, more severe than in human studies. In contrast to the reduction of pre(AGEs) by physical activity found in the abovementioned studies, a clinical study with middle-aged overweight and obese men did not show any effect on serum AGEs after a 3-month aerobic moderate intensity exercise intervention[25]. We previously found, using state-of-the-art measurements of dicarbonyls and AGEs with UPLC tandem MS, that lifelong exercise training was linked to reduced plasma levels of the AGE MG-H1 and reduced dicarbonyl stress while plasma CML and CEL were higher[23]. These mixed outcomes of physical activity on (pre)AGEs are presumably due to heterogeneities between study populations, duration of intervention, the intensity of physical activity and techniques used to measure AGEs. Our current study adds to previous work by including a large array of protein-bound and free AGEs as well as the major dicarbonyls, providing a relatively large sample size of older adults and applying a HIIT intervention in the context of plasma glycation for the first time.

This study has a few important implications. First, the current study suggests that it is unlikely that glycation in older individuals can easily or quickly be targeted by interventions that improve physical fitness through HIIT. This is relevant as we previously

found that caloric restriction is a potent intervention to reduce dicarbonyl stress[37]. In addition, scavenging compounds that reduce glycation may therefore still be needed to lower glycation in-vivo. This is challenging, as some of these compounds such as aminoguanidine were either linked to potential toxicity, or had limited efficacy when tested in humans[38]. However, some compounds such as pyridoxamine, carnosine or carnosinol and the trans-resveratrol/hesperetin co-formulation remain under active investigation with potential efficacy and a more favorable pharmacologic profile[39-41].

This study has several limitations. We showed previously that free plasma AGEs are associated with dietary intake of AGEs[36] and in our study, we cannot exclude the confounding influence of dietary AGEs. Moreover, we do not know if the current findings were in any way confounded by caloric intake. Furthermore, our intervention, although quite intense for older individuals, had a duration of 12 weeks, and longer interventions may have had more profound effects on plasma glycation markers. Finally, we cannot rule out that exercise lowers tissue AGEs and dicarbonyls, but that this change is not reflected by plasma measurements. It should also be noted that our results cannot be generalized to a younger population.

Clinical perspectives

1. The effect of physical activity on AGE accumulation is still unknown and human studies linking exercise training with dicarbonyl stress or AGEs have yielded conflicting results.
2. Plasma markers of glycation are not increased in sedentary individuals or lower in physically active older individuals. Protein-bound CML levels were positively associated with VO_2 max, and appeared to be associated with physical fitness.
3. These findings imply the need for other approaches, such as pharmacological agents, directed at reducing age-related diseases and cardiovascular risk through reduction of the glycation pathway.

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Supplemental materials

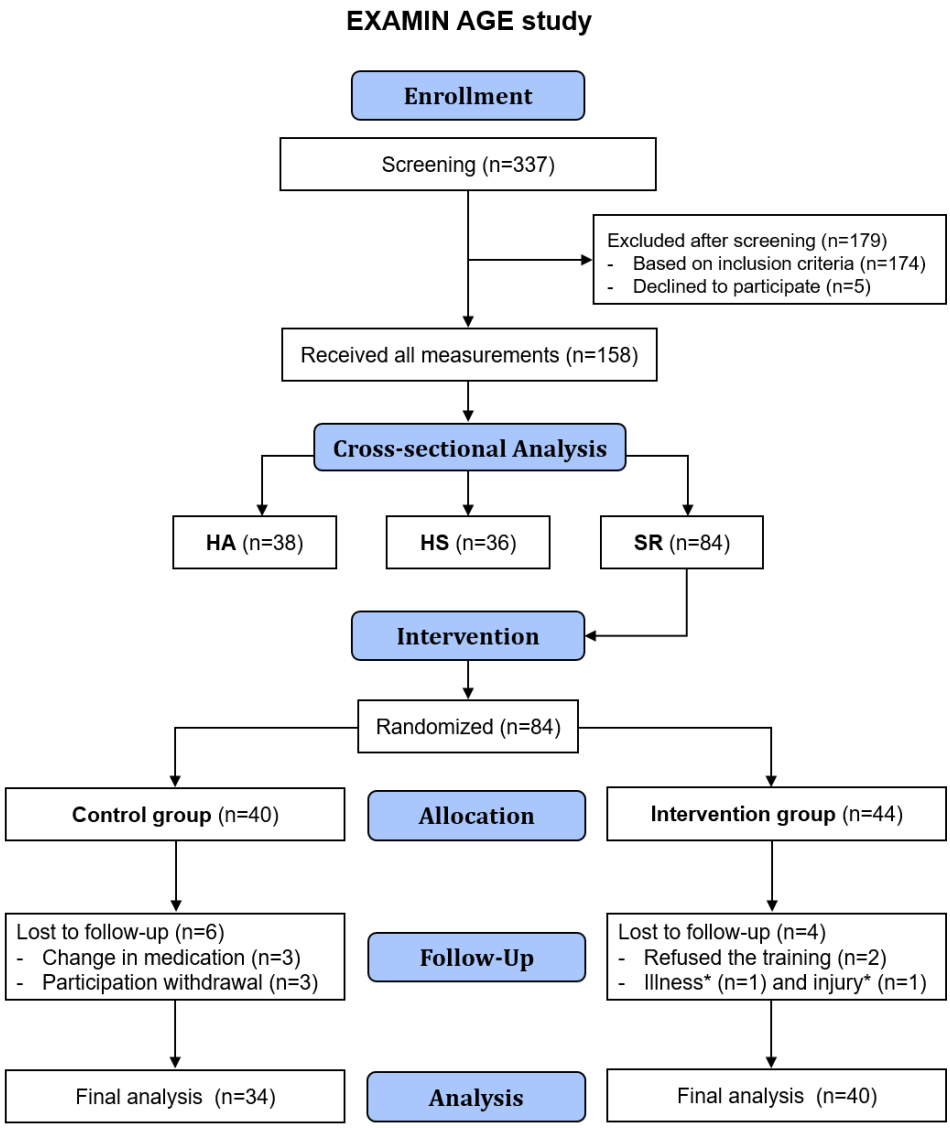

Figure S3.1 **Flow chart of the EXAMIN AGE study.** HA: healthy older active, HS: healthy older sedentary, SR: older sedentary at risk.
* Not due to the intervention.

Table S3.1 Risk factor distribution in the sedentary at risk group.

	n	%
Obesity	71	85
High Blood Pressure	62	74
SBP ≥ 140 mmHg or DBP ≥ 90 mmHg (24 h)	30	36
Antihypertensive medication	41	49
Dyslipidemia	47	56
LDL > 4.9 mmol/L	8	10
HDL < 1.0 mmol/L (male) or <1.2 mmol/L (female)	18	21
Triglycerides > 1.7 mmol/L	30	36
Cholesterol lowering medication	16	19
Hyperglycemia	36	43
Fasting glucose ≥ 5.6 mmol/L	36	43
Glucose-lowering medication	12	14
Smoking	28	33

The SR group was aged 50-80 years, and had at least two additional cardiovascular risk factors (being either obesity, elevated blood pressure, elevated triglyceride or LDL levels, decreased HDL levels, elevated plasma glucose, or current smoking).

Chapter 4

Quercetin but not epicatechin decreases plasma concentrations of methylglyoxal in a randomized double-blind, placebo-controlled, crossover trial with pure flavonoids

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Abstract

Background

Methylglyoxal (MGO) is the most potent precursor of advanced glycation endproducts (AGEs). MGO and AGEs have been associated with diabetes, its complications, and other age-related diseases. Experimental studies have shown that the flavonoids quercetin and epicatechin are able to scavenge MGO and lower AGE formation.

Objective

Data about the effects of these flavonoids on MGO and AGE levels in humans are not yet available. We therefore investigated the effect of quercetin and epicatechin on the concentrations of MGO and AGEs in a post-hoc analysis.

Methods

Thirty-seven apparently healthy non-smoking adults with a systolic blood pressure between 125 and 160 mmHg at screening were included in a randomized double-blind, placebo-controlled, crossover trial. Participants ingested (-)-epicatechin (100 mg/day), quercetin 3-glucoside (160 mg/day) or placebo capsules for periods of 4 weeks, separated by 4-week washout periods. Fasted blood samples were collected at the start and end of each intervention-period. Liquid chromatography tandem mass spectrometry was used to determine plasma levels of the dicarbonyl compounds MGO, glyoxal (GO) and 3-deoxyglucosone (3DG) and free and protein-bound AGEs. Gene expression of glyoxalase 1, the enzyme involved in the degradation of MGO, was determined by either microarray or qRT-PCR.

Results

The treatment effect (□treatment - □placebo) of quercetin on MGO was -40.2 nmol/L (95% CI: -73.6, -6.8; P = 0.019), a decrease of 11% from baseline values, while GO, 3DG and free- and protein-bound AGEs did not change significantly. Epicatechin did not affect the concentrations of dicarbonyls and free- and protein-bound AGEs. We did not find a significant change in expression of glyoxalase 1.

Conclusion

In apparently healthy (pre)hypertensive men and women, quercetin but not epicatechin decreased plasma MGO levels. Quercetin may potentially form a new treatment strategy for diseases in which MGO plays a pivotal role. The trial was registered at clinicaltrials.gov as NCT01691404.

Introduction

Protein glycation and the formation of advanced glycation endproducts (AGEs) were originally viewed as post-translational modifications of proteins that accumulate slowly on extracellular and long-lived proteins throughout life. AGEs have been implicated in the pathogenesis of age-related diseases, in particular diabetes[1]. However, so far we do not have intervention strategies in humans to inhibit the formation of AGEs.

In addition to the slow formation of AGEs, glycation adducts are also formed in a fast manner on intra- and extracellular proteins by dicarbonyl compounds including methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3DG). The highly reactive MGO, which is mainly generated as a by-product of glycolysis, is a key compound involved in the very fast generation of glycation adducts on proteins[2,3]. MGO reacts primarily with arginine residues of proteins, forming the major AGE methylglyoxal-derived N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1)[4], and with lysine residues forming N^{ε}-(1-carboxyethyl)lysine (CEL). The glyoxalase system, with glyoxalase 1 (GLO1) as the key enzyme, converts MGO to D-lactate[5]. MGO and MGO-derived AGEs have been shown to induce a number of deleterious effects in cellular systems including inflammation and endothelial dysfunction[6,7]. In accordance, increasing evidence has identified MGO and the glyoxalase system as an important contributor in the development of diabetes and its complications[1] and other age-related diseases such as hypertension[8], atherosclerosis[9], cancer[10], and neurodegenerative disorders[11]. Therefore inhibition of MGO formation or neutralization of MGO may be a potential novel target for therapy against the development of such age-related complications.

Several scavengers of MGO and other reactive dicarbonyl species have been described, including aminoguanidine[12] and alagebrium[13]. However, because of safety concerns and a lack of efficacy in human clinical trials, use of these compounds has been discontinued in clinical studies[1].

It is increasingly apparent that pharma-nutritional supplements can modulate cardiovascular risk. In this regard, flavonoids are of particular interest. It has been shown in multiple experimental studies that the flavonoid quercetin, and to a lesser extent epicatechin, are able to scavenge MGO, and can potentially inhibit AGE formation[14-21]. However, these in-vitro studies are severely limited by the fact that they do not take into account the bioavailability of flavonoids, and some metabolites of quercetin and epicatechin may have distinct biological actions. Evidence from controlled human studies on the effects of quercetin and epicatechin on dicarbonyl compounds and AGEs is lacking.

In this post-hoc analysis of a human randomized controlled trial with apparently healthy (pre)hypertensive adults[22], we examined the effect of epicatechin and quercetin on the levels of dicarbonyl compounds, AGEs, and on the expression of GLO1.

Methods

Study population and design

The research design of this study has been described previously[22]. In short, 37 apparently healthy non-smoking men and women aged 40–80 years old were recruited from Wageningen and surroundings, in the Netherlands. Inclusion criteria included a BMI (in kg/m^2) between 20 and 40 and SBP between 125 and 160 mm Hg on 2 separate occasions. Participants were excluded in case of chronic disease, use of medication or prescribed diets, unstable bodyweight, moderate to vigorous physical activity ≥10 h/wk, and pregnancy or lactation. Written informed consent of all participants was obtained; the study was approved by the Medical Ethics Committee of Wageningen University (NL40772.081.12). The trial was registered at clinicaltrials.gov as NCT01691404.

This study with three interventions in a crossover design, was conducted in a randomized, double-blind, placebo-controlled manner between October 2012 and March 2013. After enrolment, subjects were randomly allocated into one of 6 different intervention sequences using computer-generated block-randomization. Participants ingested equimolecular amounts of (-)-epicatechin (100 mg/day = 345 µmol/day) and quercetin 3-glucoside (160 mg/day = 345 µmol/day) and placebo capsules for periods of 4 weeks, in random order and separated by 4-week washout periods. The dosage of epicatechin chosen was in line with the amount of epicatechin present in previous cocoa/chocolate intervention studies (46–107 mg/d)[23-26]. For quercetin an equimolecular dose was chosen. Subjects were asked to avoid consumption of flavonoid-rich foods (cocoa, tea, apples, onion, and red wine) during the study. The evening before each measurement day, subjects consumed a standardized low-flavonoid meal provided by the research team (boiled potatoes with a meatball and spinach). Fasted blood samples were collected at the research center at the start and end of each intervention-period. Procedures of office blood pressure measurements, analysis of blood glucose, HDL, LDL, total cholesterol, and triglycerides were performed as previously decribed[22].

Measurement of plasma dicarbonyls and AGEs

At the beginning and end of each intervention period, fasting venous blood samples were collected, centrifuged and plasma was stored at -80°C until analysis. Plasma levels of dicarbonyls and AGEs were measured in EDTA plasma samples.
Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to determine the plasma levels for MGO, GO, and 3-DG as previously

described[27]. Plasma levels of the free and protein-bound AGEs MG-H1, CEL, and N^{ε}-(carboxymethyl)lysine (CML) were determined with UPLC-MS/MS. Protein-bound pentosidine was determined with high performance liquid chromatography with fluorescent detection[28].

Glyoxalase 1 expression

Fasting PBMCs were isolated using BD Vacutainer Cell Preparation Tubes. RNA was isolated from the PBMC samples and gene expression of GLO1 was measured before and after 4 weeks supplementation of quercetin, epicatechin and placebo. Gene expression of GLO1 in the epicatechin and placebo intervention was assessed by microarray analysis (as a part of a whole genome analysis), while the gene expression of GLO1 in the quercetin and placebo intervention groups was determined by qRT-PCR.

For the microarray analysis, RNA was isolated (RNeasy Micro kit, Qiagen, Venlo, the Netherlands), quantifed (Nanodrop ND 1000, Nanodrop technologies, Wilmington, Delaware USA) and integrity was checked by an Agilent 2100 Bioanalyser with RNA 6000 microchips (Agilent Technologies, South Queensferry, UK). Samples were included for microarray analysis if the RNA integrity number (RIN) was >7. Total RNA was labelled using a one-cycle cDNA labelling kit (MessageAmpTM II-Biotin Enhanced Kit; Ambion Inc, Nieuwekerk a/d IJssel, Netherlands) and hybridised to GeneChip® Human Gene 1.1 ST Array targeting 19,738 unique gene identifiers (Affymetrix Inc. Santa Clara, CA). Microarray analysis and calculation of expression levels was performed according to manufacturer's instructions, as described previously[29].

For the qRT-PCR measurement, RNA was isolated from PBMC's using Trizol reagent (Ambion) and cDNA was synthesized using the iScript cDNA synthesis kit (170-8891; Bio-Rad, Hercules, USA), both according to manufacturer's instructions. Gene expression was determined on a CFX96 Touch with CFX manager software (Biorad) using IQ SensiMix SYBR master mix (Bioline, London, UK). mRNA expression levels were normalized to the geometric mean of reference genes ribosomal protein S18 (RPS18) and large ribosomal protein (RPLPO) (Supplemental Table S4.1). The ΔΔCT method was used to calculate expression levels[30] and data are expressed as normalized gene expression levels relative to control.

Statistical analysis

Statistical analyses were performed according to a pre-defined plan, previously described by Dower et al.[22]. As outcomes, changes in AGEs and dicarbonyl content were measured, and were observed to be normally distributed. The difference between the change in AGE and dicarbonyl content during each 4-wk treatment period (Δtreatment)

and the change during the 4-wk placebo period (Δplacebo) was calculated. This difference (Δtreatment - Δplacebo) was defined as the treatment effect of epicatechin and quercetin. A linear mixed model approach was used to evaluate the treatment effects in this crossover study. Treatment and period were set as fixed effects and subject was set as random effect. Furthermore, the interaction between treatment and period was tested, and so was the previous treatment to investigate possible carry-over effect. There was no significant interaction between treatment and period for both AGEs and the dicarbonyls. No carryover effect was apparent and therefore previous treatment was not included in the final model.
Compound symmetry was used as covariant structure, for most models this resulted in the best fit according to the likelihood ratio test. Treatment effects are expressed as least squares mean with 95% CIs.
As explanatory variable, the change over time in GLO1 expression, due to either intervention (Δ quercetin, Δ epicatechin, or Δ placebo) was assessed using a paired samples t-test. The difference between the change during both flavonoid interventions and the placebo intervention (Δ quercetin vs. Δ placebo, and Δ epicatechin vs. Δ placebo) was analyzed in the same way.
Statistical significance was set at a 2-sided level of 0.05. SPSS Statistics 23 was used for all analyses.

Results

In total, 37 participants were included in the study and 35 participants completed the placebo, quercetin and epicatechin treatment. The mean age of the study population at the start of the study was 66.4 ± 7.9 years. Participants had a mean blood pressure of 129/75 mmHg, 22% of the study population was hypertensive (SBP≥140 and/or DBP≥90 mmHg), and 62% had a BMI larger than 25. Baseline characteristics are summarized in Table 4.1.

Table 4.1 Baseline characteristics of 37 healthy subjects randomly allocated at the start of the study.

Characteristic	Mean ± SD
Age (yrs)	66.4 ± 7.9
BMI (kg/m^2)	26.7 ± 3.3
Office SBP (mmHg)	129 ± 14.1
Office DBP (mmHg)	74.8 ± 9.8
Plasma glucose (mmol/L)	5.71 ± 0.65
Fasting serum lipids (mmol/L)	
Total cholesterol	5.63 ± 0.90
LDL cholesterol	3.49 ± 0.80
HDL cholesterol	1.54 ± 0.41
Triglycerides	1.29 ± 0.57
Dicarbonyls	
MGO (nmol/L)	380 ± 62.4
GO (nmol/L)	959 ± 189
3-DG (nmol/L)	1220 ± 205
AGEs	
CML (nmol/L)	109 ± 29.2
CEL (nmol/L)	59.1 ± 14.2
MG-H1 (nmol/L)	231 ± 194
PB CML (nmol/mmol lysine)	108 ± 24.7
PB CEL (nmol/mmol lysine)	38 ± 10.7
PB MG-H1 (nmol/mmol lysine)	272 ± 58.3
PB Pentosidine (nmol/mmol lysine)	0.58 ± 0.29

Data are given as mean ± SD, n=37. BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone, CML: N(ε)-(carboxymethyl)lysine, CEL: N(ε)-(1-carboxyethyl)lysine, MG-H1: N(δ)-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine, PB: protein bound AGEs.

The treatment effect of quercetin supplementation on plasma MGO levels (Δquercetin - Δplacebo), was a significant decrease of plasma MGO in the quercetin treatment compared to the placebo treatment (-40.2 nmol/L; 95% CI: -73.6, -6.8; p=0.019) (Table 4.2). This was a reduction of 10.6% of the average baseline MGO value (380 nmol/L) from the study population. GO, 3DG, free AGEs and protein-bound AGEs did not change significantly by quercetin treatment.

Epicatechin supplementation did not affect plasma levels of MGO, GO, 3DG or AGEs.

The expression of GLO1 did not change significantly during treatment with either quercetin or epicatechin. The mean difference in relative GLO1 expression (fold change) between the quercetin and placebo intervention (Δ quercetin vs. Δ placebo) as measured by qRT-PCR was -0.07 (95% CI: -0.73, 0.58; p=0.819). The mean difference in signal-to-log ratio (SLR) change between the epicatechin and placebo intervention as measured by micro-array (Δ epicatechin vs. Δ placebo) was -0.01 (p=0.79).

Table 4.2 Effects of (-)-epicatechin and quercetin-3-glucoside supplementation on levels of dicarbonyls and free and protein-bound AGEs.

	(-)-Epicatechin (n=35)		Quercetin-3-glucoside (n=35)	
	Treatment effect (95% CI)	p value	Treatment effect (95% CI)	p value
MGO (nmol/L)	-1.13 (-34.4, 32.1)	0.946	-40.2 (-73.6, -6.82)	0.019
GO (nmol/L)	17.1 (-77.8, 112)	0.720	27.5 (-67.5, 123)	0.565
3-DG (nmol/L)	3.11 (-44.9, 51.1)	0.897	6.89 (-41.2, 55)	0.776
CML (nmol/L)	9.14 (-4.15, 22.4)	0.176	7.66 (-5.68, 21)	0.258
CEL (nmol/L)	4.38 (-6.52, 15.3)	0.425	1.37 (-9.57, 12.3)	0.804
MG-H1 (nmol/L)	-22.3 (-140, 95.8)	0.708	-16.9 (-135, 101)	0.777
PB CML (nmol/mmol lysine)	-2.27 (-7.88, 3.34)	0.424	0.704 (-4.93, 6.34)	0.805
PB CEL (nmol/mmol lysine)	-2.30 (-7.82, 3.21)	0.407	-0,091 (-5.62, 5.43)	0.974
PB MG-H1 (nmol/mmol lysine)	5.77 (-17.6, 29.1)	0.625	-6.22 (-29.6, 17.2)	0.600
PB Pentosidine (nmol/mmol lysine)	-0.016 (-0.039, 0.006)	0.156	-0.012 (-0.035, 0.011)	0.299

Data are least square means from linear mixed model for repeated measures with compound symmetry as the covariant structure, n=35. Treatment effect = (Δtreatment - Δplacebo). Measurements of dicarbonyls and AGEs in fasting plasma samples from the start and end of every intervention period were performed as described in the methods.
MGO: methylglyoxal, GO: glyoxal, 3-DG: 3-deoxyglucosone, CML: N(ε)-(carboxymethyl)lysine, CEL: N(ε)-(1-carboxyethyl)lysine, MG-H1: N(δ)-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine, PB: protein bound AGEs.

Discussion

In the current study we found that the flavonoid quercetin decreased plasma levels of MGO in apparently healthy (pre)hypertensive adults while no significant effects were found for GO, 3-DG or AGEs. Epicatechin on the other hand did not show any significant decrease of either dicarbonyl levels or AGEs.

It has been reported that quercetin may be able to scavenge MGO and GO[14,19], and inhibit AGE formation[15,31-33]. Although anti-glycating effects of epicatechin have also been described[34,35], the MGO-trapping capacity has been found to be weaker as compared to quercetin[15,36,37]. In accordance with these in vitro experiments, we found that MGO levels were significantly decreased by quercetin, but not by epicatechin. To explain the difference in effect between quercetin and epicatechin on MGO, knowledge about the mechanisms of scavenging of MGO by flavonoids is of importance. It has been demonstrated that there are several structural requirements for flavonoids to be able to scavenge MGO and other dicarbonyls[14,38]. The major active sites of the flavonoids are on the A ring, with critical presence of a second hydroxyl group for efficient trapping of dicarbonyls. This part is identical for quercetin and epicatechin. However, at the C ring, epicatechin lacks a double bond and a ketone group which could explain why quercetin has a higher MGO trapping efficacy in comparison to epicatechin[36] and why we do see an effect of quercetin, but not of epicatechin, on MGO levels.

In addition to the potential scavenging of MGO by quercetin, an indirect effect of quercetin on the expression of GLO1 may also explain the reduction of MGO. GLO1 has an antioxidant responsive element (ARE) in its promoter, and indeed, it has been shown that activators of nuclear factor E2-related factor 2 (Nrf2) increase the expression of GLO1[39] and thereby decrease MGO levels[40], although we should emphasize that this has been tested in experimental research only and not in humans. It has been described that both quercetin[41] and epicatechin[42,43] can activate Nrf2 and that quercetin, among other flavonoids, enhances the glyoxalase pathway and consequently reduces MGO concentrations in neuron cells[44]. In our study, however, we did not find an effect of quercetin or epicatechin on GLO1 expression. In addition, we did not find any change in plasma glucose levels by these polyphenols. This strongly suggests that quercetin, with this dosage and duration as applied in our study, predominantly exerts its lowering effect on MGO levels via biochemical scavenging and not via glucose lowering or an upregulation of GLO1.

In our previous study[45] we found that supplementation of pure quercetin significantly decreased sE-selectin, IL-1β, and a z-score of inflammation markers. Since MGO is associated with expression of pro-inflammatory markers[1] and activation of NF-κβ[8], a reduction of MGO by quercetin on its turn may reduce levels of sE-selectin and IL-1β.

Although quercetin and epicatechin were administered in equimolar quantities, their individual bioavailability should be considered[46-50]. The daily dosage of (-)-epicatechin was 100 mg/day, and for quercetin 3-glucoside an equimolar dose of 160 mg/day. This is approximately ten-fold higher compared to the normal habitual intake in the Netherlands, which is 11 mg/day for epicatechin and 16 mg/day for quercetin. Dower et al.[22] showed that an acute-on-chronic administration of equal doses resulted in plasma values of about 2000 nmol/L for quercetin as well as epicatechin after 2 h of administration. However, the elimination half-lives of quercetin and epicatechin are quite different: 17 h for quercetin and 2 h for epicatechin. As a result, an ingested dose of epicatechin will be quickly eliminated from the blood in a few hours, whereas elimination of a dose of quercetin proceeds much slower, and will take a few days. Thus in addition to its structure that enables a more efficient trapping of dicarbonyls, the slow elimination of quercetin further enhances the scavenging of MGO. Cell culture experiments have also shown an intracellular uptake of quercetin[51]. Quercetin and other flavonoids are rapidly modified during first-pass metabolism. Conjugates with glucuronic acid and sulfate can be formed, while a part of these flavonoids is also methylated. These conjugated forms may also affect the bioactivity[49,52], and the trapping of dicarbonyls. It should be emphasized that many experimental studies with flavonoids have been performed with unconjugated quercetin and epicatechin and this may clarify differences in results of inhibition of AGE formation between experimental and in vivo

models. Therefore, the results of several experimental studies in vitro with high dosages of unconjugated flavonoids performed under non-physiological conditions should be interpreted with care.

We found a significant reduction of 10.6% of plasma MGO by quercetin. Recently our group demonstrated that MGO levels are associated with total, fatal, and non-fatal incident cardiovascular disease in type 1 and type 2 diabetes[53,54] with an observed difference in plasma MGO concentrations of ~ 5-13 % between diabetic individuals with and without cardiovascular events. In this regard, the treatment effect of quercetin in this population with a reduction of MGO of 10.6% is most likely of clinical relevance.

This study was a post-hoc analysis of a human randomized controlled trial[22] and a limitation is that this trial was not originally designed to detect an effect of quercetin and epicatechin on dicarbonyl compounds and AGEs. However, we found a significant reduction of MGO and this was in full agreement with data from in vitro experiments. Therefore, we do not think that the reduction of MGO by quercetin is a chance finding. In contrast to MGO, we did not find a significant effect on the MGO-derived AGEs MG-H1 and CEL in plasma. These observations might be due to a lack of power, or the effects of quercetin and epicatechin in this moderately low concentration were too small to detect a potential decrease in AGEs. It should also be emphasized that we performed this study in relatively healthy individuals with a short intervention period of 4 weeks and that we therefore cannot exclude the possibility that we underestimated the long term effects of quercetin and epicatechin and that results may differ in high-risk populations such as diabetic individuals. Nonetheless, the AGE levels we report in our current study fall within a similar range as previously published in other studies that used UPLC-MS/MS to quantify AGE levels[55,56].

In conclusion, in this first human intervention study that has investigated the effects of pure flavonoids on reactive dicarbonyl compounds and AGEs, we found a significant reduction of plasma MGO levels by quercetin supplementation, but not by epicatechin supplementation. The scavenging effect of quercetin on MGO, together with its anti-inflammatory capacity, may potentially form a new treatment strategy for diseases in which MGO plays a pivotal role.

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Supplemental materials

Table S4.1 Primer sequences used for qRT-PCR.

RPS18 Human ATCCTCAGTGAGTTCTCCCG Forward
RPS18 Human CTTTGCCATCACTGCCATTA Reverse
RPLPO Human GCAATGTTGCCAGTGTCTG Forward
RPLPO Human GCCTTGACCTTTTCAGCAA Reverse
GLO1 Human GGTTTGAAGAACTGGGAGTCAAA Forward
GLO1 Human ATCCAGTAGCCATCAGGATCTTG Reverse

RPS18: Ribosomal protein S18, RPLPO: Large ribosomal protein, GLO1: Glyoxalase 1.

Chapter 5

Pyridoxamine study trial design:
The effect of pyridoxamine supplementation on vascular function and insulin sensitivity; a double-blind randomized placebo controlled trial in abdominally obese subjects

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Based on the (supplemental) protocol of chapter 6-8

Objective

The objective was to study the physiological effects of a dietary intervention with pyridoxamine dihydrochloride in abdominally obese individuals. We investigated micro- and macrovascular function, endothelial function, whole body insulin sensitivity, glucose metabolism, dicarbonyl concentrations, AGE concentrations, and a large array of blood parameters related to endocrine disease and chronic inflammation.
Below, the study protocol of this trial and a short substudy are described.

Study design

We conducted an eight-week, double blind, randomized, placebo-controlled trial with the dietary supplement pyridoxamine. The study has a parallel design comprising of three study groups. The groups consist of a placebo treatment, a 25 mg/day pyridoxamine dosage (low dosage), and a 200 mg/day pyridoxamine dosage (high dosage).
Upon completion of all baseline (pre-intervention) measurements, subjects were randomly assigned to one of the three parallel groups. We had an equal randomization ratio over the three groups and the predefined randomization process was stratified for gender and age. Afterwards, the intervention period of 8 weeks started and people received either placebo of pyridoxamine capsules. After 8 weeks the follow-up (post-intervention) measurements were performed (Figure 5.1).
In the end 112 individuals with abdominal obesity were randomized, 37 to placebo treatment, 38 to the low dosage group, and 38 to the high dosage group (Figure 5.2 Flowchart).

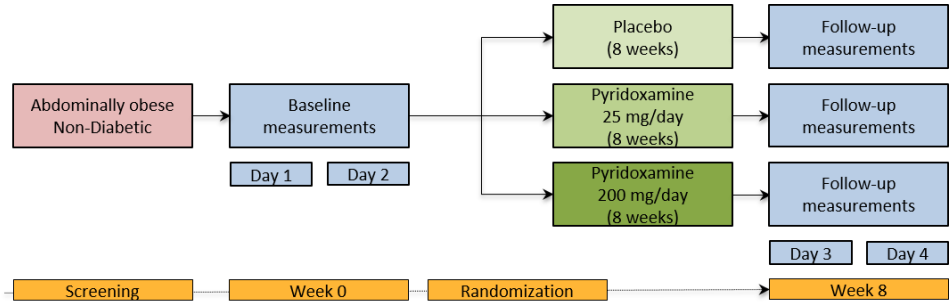

Figure 5.1 Study design.

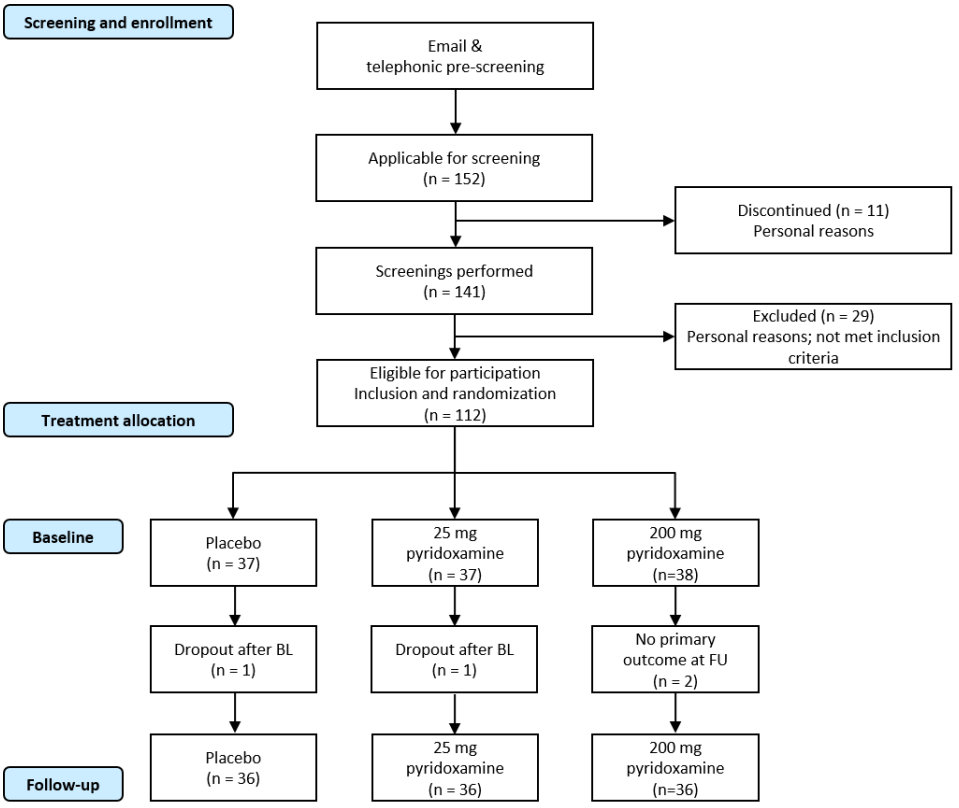

Figure 5.2 Flow diagram of study participants from the pyridoxamine trial.
After screening, 112 individuals were eligible for participation, 110 completed the study and 108 completed all primary outcome measurements.
Baseline measurements were performed in 112 abdominally obese men and women. 37 individuals were randomized to the placebo group, 37 individuals were randomized to the low dosage, 38 individuals were randomized to the high dosage. Two individuals dropped out, the first because of personal reasons and the second due to medical reasons (not related to the intervention). Due to practical complications, the primary outcome measurement was not completed in two people of the high dosage group. The study was stopped after a minimum of 108 completed follow-up measurements. BL = Baseline measurements, FU = follow-up measurements.

Study population

The study population after screening consisted of 112 abdominally obese individuals, male and female, between 18 and 75 years of age. Volunteers were typically Caucasian individuals recruited from Maastricht and the surrounding area in the Netherlands. The inclusion criteria were based on waist circumference. Minimum waist circumference for

men was 102 cm and for women 88 cm. To reach the amount of people meeting the required inclusion criteria, we performed an indefinite number of pre-screenings of applicants by phone, and physically screened 141 applicants.

Inclusion criteria

In order to be eligible for participation, men and women must meet the following criteria:

- Abdominal obesity: Waist circumference for men should be above 102 cm and for women above 88 cm
- Light to moderate skin colour (commonly Caucasian) is required for skin fluorescence and capillary microscopy measurements.
- Aged 18-75 years

Exclusion criteria

Potential participants meeting any of the following criteria, or having any of the following illnesses, were excluded from participation:

- Diabetes mellitus (i.e. using anti-diabetic medication, fasting glucose >7.0 mmol/L, HbA1c >6.5%).
- Active or history of cardiovascular disease (e.g.: diagnosed stroke, coronary artery disease, peripheral vascular disease, congestive heart failure, cardiac shunts, cardiac surgery, pulmonary hypertension, cardiac arrhythmias, family history of cardiac arrhythmias or sudden cardiac death)
- Hyperlipidemia, defined as serum total cholesterol >8 mmol/L or TG >4 mmol/L. (Lipid lowering medication was allowed if there was no current hyperlipidemia or other related disease)
- Use of medication known to influence glucose metabolism or vascular function (e.g. glucocorticosteroids, NSAID's)
- Higher grade hypertension: blood pressure >179 mmHg SBP and/or >109 mmHg DBP. (Antihypertensive medication was allowed.)
- Smoking: smokers were excluded if they smoked >10 cigarettes per day. (In the end, no smokers were included in this study.)
- High alcohol usage (>4 units/day) or drug abuse
- Known allergic reaction to ultrasound contrast-agent
- Diagnosed pulmonary or inflammatory disease with the exception of common allergies
- Kidney failure or electrolyte disorders
- Use of dietary supplements or an investigational product within the previous month

- Unstable body weight (no drastic changes in life style before or during the intervention were allowed, this means no weight gain or loss >3 kg in the previous two months)
- Pregnancy or lactation
- Changes in use of oral anticonceptiva or IUD (12 weeks prior or during the intervention)
- Unwillingness to give up being a blood donor (donating blood 8 weeks prior to the start of the study or during the study was not allowed)
- Insufficient knowledge of the Dutch language

Recruitment, consent and screening visit

Participants were recruited through local paper media, online social media, recruitment websites, and general practitioners. During this first contact, a pre-screening was performed to investigate if the individual met the requirements for this study. If the subject appeared eligible to participate, he or she received the written information and could contact an independent physician for further information. When a person could be included, informed consent was signed and an extensive screening visit took place.
Medical history was discussed, a medical doctor performed heart and lungs auscultation, anthropometric measurements were taken, and office blood pressure was determined. Finally, a fasting blood sample was drawn to determine lipid profile, fasting glucose, HbA1c, and kidney function. If one or more exclusion criteria were met, participants were excluded from the study and referred to visit their general practitioner or a medical specialist, if needed.

Study days and outcomes

All study measurements took place at Maastricht University Medical Centre (MUMC+), in the vascular circulation lab of the Internal Medicine department and the school for cardiovascular research of Maastricht University (CARIM).
A total of 5 visits were scheduled: a screening visit, two experimental test days before, and two identical test days after the PM intervention. On the first test day the oral glucose tolerance test, anthropometric measurements and the FFQ were performed. On the second day, the primary outcomes insulin sensitivity and microvascular function were assessed. In addition the following panel of measurements were performed: skin flowmotion and heat-induced hyperemic response, brachial artery flow-mediated vasodilation, measurement of glycocalyx thickness, and calculation of the fatty liver

index and eGFR. Furthermore, we measured pyridoxamine metabolites, dicarbonyl concentrations, AGE concentrations, skin autofluorescence, and markers of endothelial dysfunction and low-grade inflammation.
Venous catheters were fitted at the start of the study days. Between the two days a 24h urine collection was performed and 24 hour blood pressure was monitored with a Mobil-O-Graph monitor (Mobil-O-Graph, APC Cardiovascular, Cheshire, United Kingdom). All measurements were conducted by the same investigator in a temperature-controlled room (24°C) after a 12-hour overnight fast and a short acclimatization period.

First study day: Oral glucose tolerance test

Glucose tolerance and β-cell function were assessed with a two-hour seven-sample oral glucose tolerance test (OGTT). The OGTT took place in the morning after an overnight fast, in a resting supine position after minimal 30 minutes of acclimatisation. Individuals received an intravenously placed catheter (Vasofix® Safety, Braun, Melsungen, Germany) and drank a mixture of 75 grams of anhydrous glucose dissolved in water (82.5 grams of glucose monohydrate). Blood samples were taken in the fasting state and 15, 30, 45, 60, 90, and 120 minutes after the glucose drink.

Second study day: vascular measurements and insulin clamp

To study the effects of pyridoxamine, a wide array of vascular measurements were performed in the vascular circulation lab, which had a controlled temperature of 24°C. The study day protocol started with anthropometric measurements. Afterwards, subjects received two intravenously placed catheters (Vasofix® Safety, Braun, Melsungen, Germany) at the left arm, one near the antecubital fossa (for infusion purposes) and one near the hand (for blood sampling). Blood samples were collected in the fasting state.
After an acclimatization period of 30 minutes during which skin autofluorescence was measured, blood pressure and heart rate measurements were completed (Omron, Hoofddorp, the Netherlands). Subsequently macrovascular measurements were performed, including pulse wave analysis (PWA), determination of carotid-femoral pulse wave velocity (PWV), carotid distensibility and intima-media thinkness measurements. Next, the flow mediated vasodilation (FMD) response was recorded.
After another acclimatization period of 20 minutes in supine position, microvascular measurements were conducted, starting with sublingual imaging of the endothelial

glycocalyx, nailfold capillary videomicroscopy, skin microvascular flowmotion analysis, and heat-induced hyperaemia response. Then, total blood flow to the forearm was measured and contrast-enhanced ultrasound (CEUS) of the forearm skeletal muscle was performed.
After completion of these measurements, the hyperinsulinemic, euglycemic clamp was started. All microvascular measurements were performed a second time on this day, during the insulin clamp in order to study insulin-induced microvascular function. Thereafter, insulin infusion was terminated. Subjects received a standard lunch, and glucose infusion was diminished gradually. When the participant was able to maintain his or her glucose level at or above baseline values, intravenous catheters were removed. The total procedure occupied approximately 8 hours.

Ethical considerations, safety and burden

All participants were well informed on the study procedures and received written information approved by the medical ethical committee of Maastricht University. All participants signed a written informed consent form before onset of the screening procedures. The study was carried out in accordance with this protocol and in agreement with the principles stated in the Declaration of Helsinki and Good Clinical Practive guidelines. The study was registered at the ClinicalTrials.gov database with identifier number: NCT02954588.
All risks related with the study procedures were disclosed before study onset and all participants were insured according to the Dutch guidelines for clinical research. No studies have yet described any directly related side effects of pyridoxamine supplementation, while in other clinical studies higher dosages (than 200 mg per day) were used[1-4]. In multiple phase 1 and phase 2 clinical studies with pyridoxamine, adverse event rates were similar to those seen in subjects receiving placebo. The only adverse events that showed an increase over placebo were observed in the highest dose of 600 mg pyridoxamine daily[2]; these were small increases in diarrhea and constipation and this was not noticeable in the 300 mg group.
Participants were requested to contact the coordinating investigator in case of complaints that had emerged after study initiation. All adverse events reported by the subject or observed by the investigator were recorded. Study monitoring was performed by the Clinical Trial Centre Maastricht. Subjects could leave the study at any time for any reason, without consequences. The investigator could decide to withdraw a subject from the study for urgent medical reasons. All participants received a financial compensation after completion of the study.

Considering the invested time of the participants, the required intake of three capsules per day, and the maintenance of their habitual lifestyle during 8 weeks, this study demanded a considerable amount of discipline and consistency of the participants. A study day occupied approximately 8 hours. Although the number of measurements performed during a study day was large, the burden for the participants was limited during this day. The measurements were performed in a comfortable supine position and were mostly non-invasive, with the exception of two intravenous catheters. The total volume of blood that was sampled during a study day was approximately 180 ml, during the OGGT approximately 70 ml.

Randomization, stratification, blinding and treatment allocation

After baseline measurements, all subjects were randomly assigned to the placebo, low dosage, or high dosage group, with a randomization ratio of 1:1:1. This was based on a computer-generated randomization procedure. For this, an independent person working at our lab created a randomization order for each stratum using block randomization with variable block sizes. The randomization was stratified for gender and age, yielding four lists: female < 50, female ≥ 50, male <50, and male ≥50 years of age.
Every participant had a unique randomization number. To ensure the procedures were performed in a double-blinded fashion, the capsules were packed and coded (based on the same randomization list) by Lab Medisan, Heerenveen. This way the researcher presented each participant with the blinded capsules matching his/her randomization number, and both parties remained blinded throughout the study. After all data analysis were performed, the randomization codes was provided to the researcher and treatment allocation was added to the dataset.

Statistical analysis and sample size calculation

Primary study parameters were whole body insulin sensitivity**,** insulin-mediated muscle microvascular recruitment, and flow mediated dilation. Briefly, secondary study parameters include a wide range of micro- and macrovascular measurements, assessment of glucose metabolism and β-cell function, and a wide range of plasma and serum measurements described below.
The effect of the intervention, being the relationship between the change in outcome and the study group, was assessed by analysis of covariance (ANCOVA) with correction for baseline values. This approach is similar to multiple regression analysis with dummy coding for the treatment group variable. Additionally, potential confounding effects of

age, sex, and outcome specific variables were evaluated. A p-value ≤0.05 was considered statistically significant.
Subjects who did not complete the follow-up measurements (drop-outs) were excluded from the statistical analysis. Compliance was monitored and can be included in the final analysis, however we choose to adhere to an intention-to-treat analysis of the study. No participants were replaced as we accounted for possible drop-outs in the power calculation. Missing data were not replaced by estimates. Analysis were performed using IBM SPSS Statistics version 25.
This study was powered to detect a change in insulin sensitivity (change of 0.7 mg/kg/min), resulting in a group size of 36 individuals based on a two-tailed significance level of 0.05 and a power of 0.80. Our effect-size estimate was based on the outcomes of our previous RCT, investigating a weight loss intervention with the same outcome parameters[5]. We aimed to achieve 50% of this outcome with regard to insulin sensitivity and insulin mediated microvascular function. A maximum drop-out rate of 10% was anticipated, resulting in a maximum of 40 individuals per group.

Handling and storage of data

At the start of the study, all participants were assigned a personal randomization number, which was linked to their name, address, date of birth and contact information in a password-protected file. This file was only accessible to members of the research team.
For study purposes and the analysis, all source data were assembled per participant based on the randomization number. Only members of the research team and monitoring organizations have access to the study data, this includes members of the assessment committee. Data will be preserved 15 years after the last measurements of the study. Blood samples and every form of body material will also be stored using the same code.

Intervention

Pyridoxamine and placebo supplementation

The 25 mg/day PM dosage resembles a normal and low food supplement dosage while the 200 mg/day dosage is included to study physiological effects. PM dosages and placebo were provided in the form of capsules, indistinguishable from one another except for the randomization number on the package. PM is a water-soluble vitamer

with a rapid uptake and metabolization. To ensure a steady plasma concentration during the day, the daily dosage was supplied as three capsules per day. The high dosage group consumed three capsules of each 66.7 mg PM, to achieve the total daily dose of 200 mg PM. The low dosage group consumed three capsules of each 8.3 mg PM, to achieve the total daily dose of 25 mg PM. The placebo group consumed three capsules of potato starch. Both PM and placebo capsules were made of cellulose. Capsules were taken three times per day shortly before or during the meal.

Compliance

The coordinating investigator contacted the participants by telephone during the intervention period to verify compliance. In addition, to evaluate compliance, remaining capsules were collected and counted after study completion. Moreover, PM metabolites were measured in fasting plasma and 24h urine samples of the last study day, these data were compared to our previous metabolization study[6] with the same PM supplement (substudy: see below and chapter 6).

Dietary intake and physical activity

During this intervention period, individuals were asked not to change their lifestyle, diet, and physical activity routines. All individuals received clear instructions on this matter and were asked to avoid excessive alcohol consumption, and use of dietary supplements of any kind, during the two-month period. Individuals were also specifically asked to avoid the intake of certain food products with high AGE content[7]. Individuals were asked to take note of any interfering events related to illness, medication, and physical activity. Furthermore, all individuals completed a food frequency questionnaire (FFQ) to evaluate their habitual diet and average AGE intake. Average AGE intake estimate was based on dietary AGE concentrations previously determined in a large food database[7].
Prior to screening and testing days, individuals were required to adhere to an overnight fast. Coffee, tea, and chocolate were not allowed 12 hours prior to measurements, alcohol was not allowed 24 hours prior to measurements, and heavy physical exercise was not allowed 48 hours prior to measurements. Individuals consumed only water on the morning of the test days. To standardize activity as much as possible, individuals were asked to travel to the hospital by public transportation or car.

Measurements

Hyperinsulinemic, euglycemic clamp

Insulin sensitivity was determined with the euglycemic, hyperinsulinemic clamp method. The first 10 minutes of the clamp, insulin (Novorapid, Novo Nordisk, Bagsværd, Denmark) was infused at a rate of 2 mU kg^{-1} min^{-1}, where after the rate was adjusted to 1 mU kg^{-1} min^{-1} for the remainder (~150 min) of the clamp. Normoglycemia was maintained by adjusting the infusion rate of a 20% D-glucose intravenous solution. Since the glucose infusion rate during a hyperinsulinemic euglycemic clamp usually takes 90 minutes to fully stabilize, whole body glucose uptake was calculated from the infusion rate during the last hour of the clamp (90-150 min) and expressed per kg body weight (GIR) and expressed per unit of plasma insulin concentration measured in this time interval (M/I-value). When the glucose infusion rate was stabilized (around 90 min), all microvascular measurements were repeated in a state of hyperinsulinemia.

(Plasma) biomarkers

Plasma biomarkers of endothelial dysfunction, low-grade inflammation, insulin, and c-peptide were assessed using a validated and commercially available multi-array detection system, based on electro-chemiluminicence technology and ELISA. Soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), soluble E-selectin (sE-selectin), C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), serum amyloid A (SAA), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), adiponectin, leptin, insulin, and c-peptide were measured in EDTA plasma samples with commercially available 4-plex sandwich immunoassay kits (Meso Scale Discovery (MSD), Rockville, Maryland, USA), as described previously[8]. Von Willebrand factor (vWf) was measured in citrate plasma with sandwich ELISA (Dako, Glostrup, Denmark). Plasma concentrations of vWf were expressed as a percentage of vWf detected in pooled citrated plasma of healthy volunteers. Creatinine, Gamma-GT, total cholesterol, HDL-cholesterol, triglycerides, HbA1c, and fasting-insulin were determined in serum samples at the Central Diagnostic Laboratory of the MUMC+. LDL-cholesterol was calculated using the Friedewald formula[9]. Plasma glucose levels during the oral glucose tolerance test and insulin clamp were determined with a YSI2300 glucose *analyzer* (*YSI*, Yellow Springs, Ohio, USA).
Furthermore, in whole blood samples we performed an immune cell differentiation test measuring the amount of circulating leucocytes, segmented granulocytes, lymphocytes, monocytes, eosinophils, and basophils.

Fatty liver index and eGFR

Liver and kidney function was assessed based on plasma and urine markers. The fatty liver index (FLI) was calculated using waist circumference, BMI, plasma triglyceride, and plasma gamma-glutamyl-transferase (GGT)[10]. Kidney function was assessed using the CKD-EPI creatinine equation for the estimation of the glomerular filtration rate (eGFR)[11].

Dicarbonyls and AGE measurements in plasma and urine

Levels of dicarbonyls and AGEs were measured in EDTA plasma samples. EDTA tubes were centrifuged at 1300 × g for 15 min at 4°C to obtain plasma. The supernatants were transferred into 1.5 ml Eppendorf tubes and stored at -80°C until further analysis. Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to determine the plasma levels for MGO, GO and 3-DG, as previously described[12]. Plasma levels of the free and protein-bound AGEs Nε-(carboxymethyl)lysine (CML), Nε-(1-carboxyethyl)lysine (CEL), and Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), were determined with UPLC-MS/MS, as previously described[13]. Coefficients of variation for all MS/MS analyses were below 10%. We calculated a combined Z-score of all plasma AGEs.

Skin autofluorescence

Skin Autofluorescence (SAF), an estimate of tissue AGEs, was measured with the AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands), as described in previous publications([14,15]. The AGE Reader is a desktop device that uses the characteristic fluorescent properties of certain AGEs to estimate the level of AGEs accumulation in the skin. In the present study, a series of three consecutive measurements were carried out.

Muscle microvascular blood volume (MBV) and insulin-mediated muscle microvascular recruitment (IMMR)

As a measure of microvascular function, insulin-mediated muscle microvascular recruitment (IMMR) was assessed by means of contrast-enhanced ultrasound (CEUS) using a Toshiba AplioXG ultrasound device. Both muscle microvascular blood volume and perfusion were measured during continuous intravenous administration of sulfur hexafluoride gas-filled microbubbles, before and during hyperinsulinemia, as previously described[5].

The ultrasound transducer was fixed in a custom stand, to image the flexor muscles of the right forearm. Microbubbles (SonoVue®, Bracco diagnostics, the Netherlands) were

delivered intravenously in the left arm, at a constant rate. After three minutes, when a steady state of microbubbles is achieved, the ultrasound measurements were acquired and after 6 minutes, infusion of SonoVue® was stopped. Six clips with real-time replenishment curves of thirty seconds each were recorded. The second set of measurements was performed during the steady state of the insulin clamp.
From the ultrasound recordings, replenishment curves were generated and analyzed offline in a blinded fashion after completion of the trial using CHI-Q software (Toshiba). IMMR was calculated as the relative increase (%) in muscle microvascular blood volume during hyperinsulinemia.

Capillaroscopy

Nailfold capillaries were visualised by intravital videomicroscopy. Nailfold skin capillaries in the dorsal skin of the third and fourth finger of the right hand were visualized by a capillary microscope with a 100 x system magnification (KK technology, UK). Capillary density was defined as the number of erythrocyte-perfused capillaries per square millimeter of skin. Post occlusive reactive hyperaemia after 4 minutes of arterial occlusion (maximum applied pressure of 250 mmHg with miniature cuff) was used to assess functional capillary recruitment. Afterwards, venous occlusion (60 mmHg of applied pressure with miniature cuff) was applied for 2 minutes, thereby exposing a maximum number of capillaries. During the measurements, the investigated finger was immobilized in a mass of clay and paraffin oil was applied to the imaged area of the nailfold to improve skin translucency and reduce skin reflections. The number of perfused capillaries in the rested state, perfused capillaries during hyperemia, capillary recruitment by hyperemia and capillary density during venous occlusion were recorded in a fasted state and during the insulin clamp.

Skin flowmotion and heat-induced hyperemic response

Periodic blood flow fluctuations, or flowmotion, arise in part from the rhythmic vessel diameter oscillations known as vasomotion. We measured flowmotion of skin microcirculation by means of laser Doppler flowmetry (LDF; PeriFlux 5000, PeriMed AB, Järfälla, Sweden) on the skin of the left forearm. LDF is based on the emission of a 780 nm wavelength laser beam, penetrating the skin and being backscattered partly by moving erythrocytes, leading to a frequency shift proportional to the velocity of the erythrocyte movement (i.e., the Doppler principle). The magnitude and frequency distribution of these changes in wavelength is directly related to the amount and velocity of the blood cells in the sampled volume. This information is converted to an electronic signal and blood flow is expressed in arbitrary units. This information can subsequently be analyzed by Fourier analyses, quantifying the relative contribution of

each frequency domain to the observed LDF signal. Low frequency signals are associated with flowmotion related to endothelial (0.01-0.02 Hz), neurogenic (0.02-0.06 Hz), or myogenic activity (0.06-0.15 Hz)[16]. In the result section we show the endothelial frequency interval of the skin microvascular flowmotion measurement.
In addition we evaluated the skin heat-induced hyperemic, endothelium-dependent, vasodilation with a second laser Doppler probe, under local heating to 44°C. Baseline skin blood flow was recorded unheated for 2 minutes, followed by rapid and local increase to 44°C probe temperature which was kept constant until the end of the registration. The heat-induced skin hyperemic response was expressed as the percentage increase in average perfusion units during the 30-minute heating phase over the average baseline perfusion units, as described previously[17].

Glycocalyx thickness

The endothelial glycocalyx thickness was determined using the GlycoScan device (GlycoCheck, the Netherlands). A side stream dark field camera (SDF) was covered by a disposable tip and positioned sublingually with the participant. Image focus, stability and pressure were adjusted to optimize image quality. Images of sufficient quality were recorded independent from operator input, using a built-in auto-capture module. The program provided by the manufacturer automatically determined the perfused boundary region (PBR) of each recorded vessel and subdivide the PBR per microvascular diameter.

Pulse wave analysis and pulse wave velocity

Pulse wave analysis was performed by applanation of a tonometer (SphygmoCor v9, AtCor Medical, West Ryde, Australia) to both the brachial artery and radial artery. Measurements were synchronized beat-to-beat and recorded in triplicate. After calibration on brachial artery pressure, the augmentation index (Aix) was automatically determined from the radial applanation tonometry recordings, computed by the SphygmoCor software.
Carotid-femoral pulse wave velocity (cfPWV) is the gold standard for the measurement of arterial stiffness. cfPWV was determined by means of the same tonometer (SphygmoCor v9, AtCor Medical, West Ryde, Australia). First, the distances between carotid artery and femoral artery, carotid artery and sternum, and sternum and femoral artery were determined using a measuring rod (Seca 207, Seca, Birmingham, United Kingdom). The pulse wave travel distance was calculated as the total straight distance between the two arterial sites. Thereafter, ECG electrodes were applied in order to identify the R-wave in the ECG complex. Pressure waveforms were determined at the right common carotid and right common femoral arteries. Difference in the time of

pulse arrival from the R wave of the electrocardiogram between the two sites (transit time) was determined. Afterwards, the program automatically calculates pulse wave velocity per successful measurements, the median of three measurements was used.

Carotid distensibility and Intima media thickness

Carotid artery distensibility and Intima media thickness was measured by means of ultrasonography in fast B-mode using a clinical ultrasound device (MyLab™70, Esaote, Maastricht, The Netherlands) and processed through a separate computer module (ARTLAB™, Esaote, Maastricht, The Netherlands).
The left common carotid artery was visualized directly proximal from the bulbus in fast brightness mode with a 7.5 MHz, 40 mm linear array ultrasound system (MyLab™ 70, Esaote, Maastricht, The Netherlands) equipped with an ARTLAB system (Esaote, Maastricht, The Netherlands). Ultrasound data and 3-lead ECG signal were simultaneously recorded during 3 consecutive 6 seconds measurements. Carotid artery diameter, distensibility, and carotid intima-media thickness measurements were simultaneously acquired. Data were stored as raw data and were analyzed offline using a custom-made MATLAB software (Dist13; Prof. A.P. Hoeks, Department of Biomedical Engineering, MUMC+, Maastricht, The Netherlands). Diastolic diameter was calculated as the difference in position between the anterior and posterior wall markers, distensibility was estimated, and the cIMT was calculated as the distance from the leading edge interface between lumen and intima to the leading edge interface between media and adventitia of the posterior wall. Subsequently, distensibility coefficient (DC), as a measure of arterial stiffness, and compliance coefficient, which represents arterial buffering capacity, were calculated.

Brachial artery flow-mediated vasodilation

Brachial artery flow-mediated vasodilation (FMD) was measured using continuous ultrasound echography as previously described[8]. During the measurement, the individual was in the supine position; the right shoulder and arm were positioned on supports for optimal comfort and stability. A clear segment of the brachial artery was visualized with a 7.5 MHz, 40 mm linear array ultrasound system (MyLab™ 70, Esaote, Maastricht, The Netherlands) in Duplex-mode to record simultaneously artery diameter and blood flow. The transducer position was fixed using a stereotactic probe holder. Consistent registration of the flow velocity and vessel wall was monitored during the recording period. A sphygmomanometer cuff was positioned on the right lower arm and rapidly inflated to 200 mmHg for 5 minutes, causing distal (forearm) hypoxia. After release, the hyperemic response takes place and brachial artery flow-mediated dilation was recorded. Duplex images were recorded continuously over a total of 15 minutes

consisting of a 5 minutes baseline reference period, 5 minutes during cuff inflation, and 5 minutes during hyperemia following rapid cuff release. Recorded videos were analyzed offline using wall detection and Doppler velocity tracing software (MyFMD; Prof. A.P. Hoeks, Department of Biomedical Engineering, MUMC+, Maastricht, The Netherlands) implemented in Matlab (The Mathworks, Natick, USA). The FMD response was quantified as the change in diameter relative to reference diameter (pre-occlusion), and additionally normalized to stimulus (i.e. the relative increase in blood flow velocity with hyperemia).

Substudy: Pyridoxamine metabolization

In light of this clinical trial, it was of major importance to evaluate the compliance. Due to the fast metabolization of pyridoxamine it was difficult to assess compliance solely based on the analysis of pyridoxamine. Therefore, additional analysis of pyridoxamine metabolites, in samples from the pyridoxamine trial, was mandatory. Necessary data on the pharmacokinetics of pyridoxamine were lacking, therefore we performed this supplementary study to the pyridoxamine trial.

Objective

The objective was to study the compliance during the clinical trial, and to assess the pharmacokinetics of pyridoxamine. After oral supplementation, we evaluated uptake, bioavailability and metabolization of pyridoxamine in plasma and urine with UPLC-MS/MS.

Study design

Five healthy volunteers, randomly recruited, were free of any signs of illness or disease, and used no medication. To study the metabolization and pharmacokinetics of PM, three protocols were used. The first protocol was a single oral dose of 200 mg PM in the morning, administered in the fasted state followed by a standardized breakfast, lunch and dinner during the day. Blood samples were drawn using an intravenous catheter every 30 minutes during the first 7 hours. After 7 hours, blood was collected every hour until 12 hours after intake of PM, followed by a sample 24 hours after intake of PM. The second protocol was a three daily oral dose of 67 mg PM, every 6 hours separated over three standardized meals during the day. This protocol was chosen to increase the plasma availability of PM during the day. The first dosage of 67 mg PM started in the morning administered in the fasted state followed by the second and third dosage around lunch and dinner time. All five participants followed this protocol and PM capsules were taken at the end of every meal. Blood samples were drawn using an intravenous catheter every hour during the first 15 hours. After 15 hours, blood was collected every 2 hours. A third protocol was implemented as control for dietary intake and possible vitamin B6 content. Two participants adhered to the exact same dietary schedule as the first two protocols but without PM supplementation. For this latter protocol, blood was collected every hour during 12 hours. Protocols 1, 2, and 3 were performed during a single day and completely standardized with regards to food intake

and physical activity. Twenty-four hour urine and fasted second void urine samples (pre- and post-oral dosage) of PM were collected. All participants received the exact same meals and drinks, at the same time points. The sample size was based on preceding results from the analytical optimization of the MS/MS quantification of vitamin B6 in plasma and urine. Since the coefficient of variation was far below 5% for pyridoxamine and most metabolites in both intra-assay and inter-assay variation, we anticipated that a sample size of five healthy subjects with no apparent metabolic disease would be sufficient to present reproducible metabolization data.

The study was conducted in accordance with the Declaration of Helsinki, approved by the Medical Ethics Committee of Maastricht University Medical Centre (NL) as a substudy to the clinical trial registered at ClinicalTrials.gov (NCT02954588). Written informed consent was obtained from all participants before study enrollment.

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Chapter 6

Quantification of the B6 vitamers in human plasma and urine in a study with pyridoxamine as an oral supplement; pyridoxamine as an alternative for pyridoxine

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Abstract

Background and aims

Vitamin B6 is involved in a large spectrum of physiological processes and comprises of the vitamers pyridoxamine (PM), pyridoxal (PL), pyridoxine (PN), and their phosphorylated derivatives including the biological active pyridoxal 5′-phosphate (PLP). While PN toxicity is known to complicate several treatments, PM has shown promise in relation to the treatment of metabolic and age-related diseases by blocking oxidative degradation and scavenging toxic dicarbonyl compounds and reactive oxygen species. We aimed to assess the metabolization of oral PM supplements in a single and three daily dose.

Materials and methods

We optimized and validated a method for the quantification of the B6 vitamers in plasma and urine using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Five healthy volunteers were recruited to study PM metabolization after a single oral dose of 200 mg PM or a three daily dose of 67 mg PM. A third protocol was implemented as control for dietary intake. Venous blood samples, twenty-four hour urine and fasted second void urine samples were collected.

Results

After a single oral dose of 200 mg PM, plasma PM increased in the first three hours to a maximum of 2324 ± 266 nmol/L. While plasma PM levels returned to baseline after ~10 hours of PM intake, PLP increased to a maximum of 2787 ± 329 nmol/L and reached a plateau. We found a small increase of PN to a maximum of 13.5 ± 2.1 nmol/L; it was nearly undetectable after ~12 hours. With a three daily dose of 67 mg PM we observed an increase and decline of plasma PM, PL, and PN concentrations after each PM intake. PLP showed a similar increase as in the single dose protocol and accumulated over time.

Conclusion

In this study we showed high plasma levels of PM after oral PM supplementation. We found steadily increasing levels of the biologically active PLP, with minimal formation of PN. The B6 vitamer PM is an interesting supplement as an inhibitor of harmful processes in metabolic diseases and for the treatment of vitamin B6 deficiency.

Introduction

Vitamin B6 is a water-soluble vitamin and it comprises the vitamers pyridoxamine (PM), pyridoxal (PL), pyridoxine (PN), and their phosphorylated derivatives pyridoxamine 5′-phosphate (PMP), pyridoxal 5′-phosphate (PLP), and pyridoxine 5′-phosphate (PNP)(Figure 6.1). Vitamin B6 is involved in developmental, metabolic and physiological processes, ranging from neuronal processes like neurotransmitter synthesis to interconversion of amino acids in energy-generating pathways[1-3]. Marginal vitamin B6 deficiency has been shown to be associated with inflammation markers, oxidative stress, cancer, cardiovascular health, and endothelial function in diabetes[4-13].

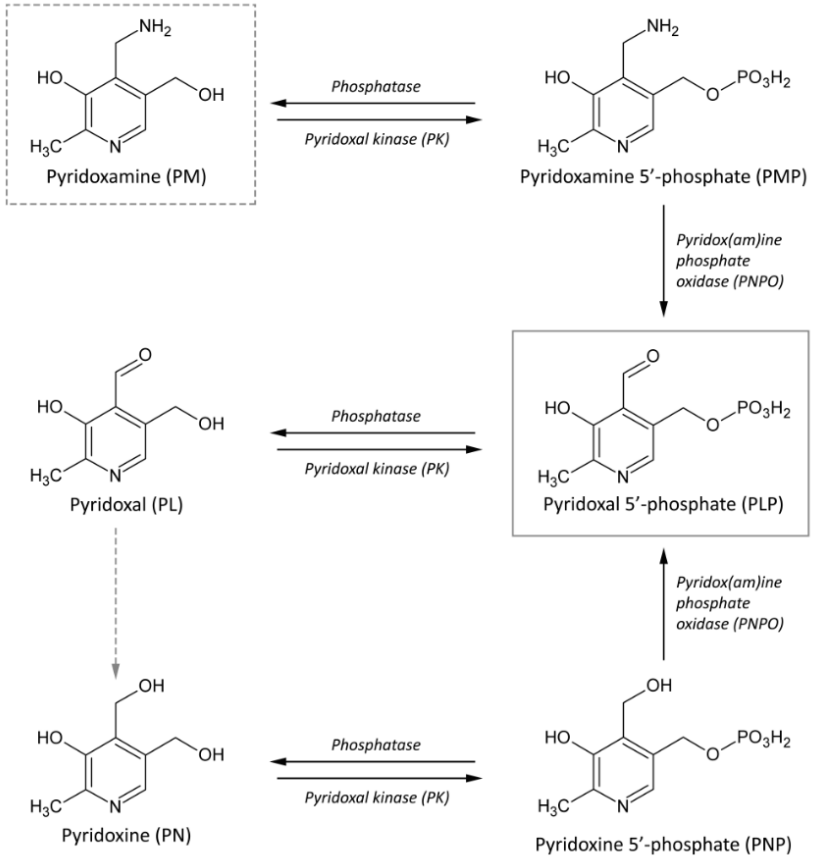

Figure 6.1 Metabolic pathways of PM and the other B6 vitamers.
PM, PL, and PN can all be converted to pyridoxal 5′-phosphate (PLP), the biologically active form of vitamin B6. PM, PL, and PN are converted to their phosphorylated forms by pyridoxal kinase (PK) that phosphorylates the 5′hydroxymethyl group. This reaction is reversible by a phosphatase. Pyridox(am)ine phosphate oxidase (PNPO) converts PMP and PNP to PLP. Recently, it has been shown that PL can also be converted to PN in humans by a pyridoxal reductase[46] (dotted grey arrow).

PN and the phosphorylated B6 vitamers are the main dietary forms of vitamin B6[1,14]. PLP is the biological active form and functions as an enzyme cofactor and regulator for many enzymatic reactions. About 4% of all human cellular enzymes are PLP-dependent. PM is a minor form of vitamin B6. It has been demonstrated that PM, but not the other B6 vitamers, can inhibit the formation of advanced glycation endproducts (AGEs)[15,16] by blocking oxidative degradation and scavenging toxic dicarbonyl compounds and reactive oxygen species[17]. Experimental studies have shown that PM improved obesity related complications[18], endothelial dysfunction[19], and kidney dysfunction[20]. In humans, several large-scale clinical trials with PM showed moderate improvements of overt diabetic nephropathy and schizophrenia[21-23].

However, little is known about PM supplementation and its metabolization in humans. To study this, we first developed a sensitive and specific analytical method to analyze B6 vitamers in plasma and urine. So far, B6 vitamers have been analyzed by several different liquid chromatography techniques with MS[24-30], electrochemical[31] and UV detection[32]. However, not all methods were validated for plasma and urine[24-26,29,32]. Based on the previously described methods for the analysis of B6 vitamers in plasma[27] and cerebrospinal fluid[24], we optimized and validated a method for the quantification of PM and its metabolites in plasma and urine using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).
We used this method to investigate PM metabolization and pharmacokinetics in a substudy of a clinical trial with oral PM supplementation (NCT02954588). Given that most available vitamin B6 supplements contain PN, and PN toxicity is known to complicate several treatments[33-37], we also addressed the important question whether the B6 vitamer PM could be a valuable therapeutic supplement for vitamin B6 deficiency by increasing PLP as the biological active form, without PN accumulation.

Materials and methods

Chemicals

Pyridoxine (PN, ≥98%), D_3-pyridoxine (D_3-PN, ≥98%), pyridoxamine-5'-phosphate (PMP, ≥98%), pyridoxal (PL, ≥99%), pyridoxal-5'-phosphate (PLP, ≥98%) and perchloric acid (PCA, 70%) were obtained from Sigma-Aldrich (Steinheim, Germany). D_3-pyridoxamine (D_3-PM) was obtained from Cambridge Isotope Laboratories (Andover, USA). D_3-pyridoxal-5'-phosphate (D_3-PLP) was obtained from Buchem (Apeldoorn, The Netherlands). Tridecafluoroheptanoic acid (TDFHA, >98%) was obtained from Alfa Aesar

(Kandel, Germany). Water and acetonitrile (ULC/MS quality) were obtained from Biosolve Chimie (Dieuze, France).

Standard and internal standard solutions

A standard working solution was prepared by dissolving PM (89.8 µmol/L), PMP (1.5 µmol/L), PLP (18.1 µmol/L), PL (17.9 µmol/L) and PN (17.9 µmol/L) in water and stored at -80°C before analysis. A six-point calibration curve was prepared by diluting the standard working solution in water with dilution factors 1, 0.75, 0.5, 0.25, 0.125 and 0. An internal standard working solution was prepared by dissolving D_3-PM (1025 nmol/L), D_3-PLP (1000 nmol/L), D_3-PL (1958 nmol/L) and D_3-PN (958 nmol/L) in 5% (v/v) PCA and stored at -80°C before analysis.

UPLC tandem MS

PM and its metabolites were chromatographically separated by ultra-performance liquid chromatography (UPLC) (Acquity UPLC, Waters, Milford, USA) and detected in ESI positive multiple reaction monitoring (MRM) mode using a Xevo TQ-XS (Waters, Milford, USA). Chromatography was done on a reversed-phase C18 column (Acquity UPLC HSS T3, 50 x 2.1 mm, 1.8 µm) with a binary gradient of 7,5 mmol/L TDFHA (solvent A) and acetonitril (solvent B) at a flow rate of 600 µl/min. A linear gradient was started at 99% solvent A, which was changed within 4 minutes to 50% solvent A. After cleaning the column with 80% solvent B during 1 minute, the column was equilibrated for 4 minutes at the initial conditions. The injection loop volume was 2 µl (1 µL partial loop with needle overfill) and column temperature was set at 45°C. Quantification of PM and its metabolites was performed by calculating the peak area ratio of each unlabeled peak area to the corresponding internal standard peak area. Electrospray ionization was done at a capillary voltage of 0.5 kV a source temperature of 150°C and a desolvation temperature of 500°C. MRM transitions for all metabolites were optimized by direct infusion via a T-piece under normal LC flow conditions. For qualitative and quantitative analysis Masslynx software (V4.1, SCN 644, Waters, Milford, USA) was used.

Method validation

Linearity was determined by adding standard working solution of PM and its metabolites to water, 2 different urine and 2 different plasma samples. Therefore, a six-point calibration curve was prepared as described previously. The peak area ratio of PM and its metabolites multiplied by the concentration of each corresponding internal standard were plotted as a function of the concentration. For evaluation of the inter- and intra-assay variation a pooled EDTA plasma and urine sample (with and without spike) were

analyzed on 3 different days (inter-assay) and 6 times on the same day (intra-assay). Recovery (%) was tested by adding standard solution of PM and its metabolites to plasma and urine samples.

Plasma sample preparation

Whole blood was collected in sterile EDTA tubes. After centrifugation, 3500 g at 4°C for 10 minutes, plasma was aspirated and stored at -80°C until analysis. Before analysis, plasma samples were thawed (if needed) and mixed thoroughly. Sixty µl plasma was mixed with 30 µl water and 60 µL internal standard working solution in an Eppendorf cup. This mixture was allowed to stand for 25 minutes at room temperature and shielded from light. Samples were mixed and subsequently centrifuged for 10 minutes at 14000 rpm at a temperature of 4°C. One µl supernatant was injected for UPLC-MS/MS analysis.

Urine sample preparation

Second void and 24h urine was collected and stored at -80°C until analysis. Before analysis, urine samples were thawed (if needed) and mixed thoroughly. Twenty µl urine was mixed with 40 µl water and 60 µL internal standard working solution in an Eppendorf cup. This mixture was allowed to stand for 25 minutes at room temperature and shielded from light. Samples were mixed and subsequently centrifuged for 10 minutes at 14000 rpm at a temperature of 4°C. One µl supernatant was injected for UPLC-MS/MS analysis. Urinary vitamer concentrations were expressed as µmol/mmol creatinine. Creatinine concentration in urine was analysed using a Beckman LX20 analyser (Beckman Coulter) based on the Jaffe reaction method[38].

Study design and population

Five healthy volunteers (3 male and 2 female; mean age: 32± 9 y, mean BMI: 21.9 ± 1.8 kg/m^2) were randomly recruited, were free of any signs of il lness or disease, and used no medication. To study the metabolization and pharmacokinetics of PM, three protocols were used. The first protocol was a single oral dose of 200 mg PM in the morning, administered in the fasted state followed by a standardized breakfast, lunch and dinner during the day (see Supplementary Table S6.1). Blood samples were drawn using an intravenous catheter every 30 minutes during the first 7 hours. After 7 hours, blood was collected every hour until 12 hours after intake of PM, followed by a sample 24 hours after intake of PM. The second protocol was a three daily oral dose of 67 mg PM, every 6 hours separated over three standardized meals during the day. This protocol was chosen to increase the plasma availability of PM during the day. The first

dosage of 67 mg PM started in the morning administered in the fasted state followed by the second and third dosage around lunch and dinner time. All five participants followed this protocol and PM capsules were taken during every meal. Blood samples were drawn using an intravenous catheter every hour during the first 15 hours. After 15 hours, blood was collected every 2 hours. A third protocol was implemented as control for dietary intake and possible vitamin B6 content. Two participants adhered to the exact same dietary schedule as the first two protocols but without PM supplementation. For this latter protocol, blood was collected every hour during 12 hours. Protocols 1, 2, and 3 were performed during a single day and completely standardized with regards to food intake and physical activity. Twenty-four hour urine and fasted second void urine samples (pre- and post-oral dosage) of PM were collected. All participants received the exact same meals and drinks, at the same time points. The sample size was based on preceding results from the analytical optimisation of the MS/MS quantification of vitamin B6 in plasma and urine. Since the coefficient of variation was below 5% for pyridoxamine and most metabolites in both intra-assay and inter-assay variation, we anticipated that a sample size of five healthy subjects with no apparent metabolic diseases would be sufficient to present reproducible metabolization data.
The study was conducted in accordance with the Declaration of Helsinki, approved by the Medical Ethics Committee of Maastricht University Medical Centre (NL), and registered at ClinicalTrials.gov (NCT02954588). Written informed consent was obtained from all participants before study enrollment.

Pyridoxamine supplement and food intake

Pyridoxamine (pyridoxamine·HCl) was obtained from Tohoku University School of Medicine (Sendai, Japan) and the supplement capsules were prepared by Medisan B.V. (Heereveen, The Netherlands). Analyses regarding the concentration, purity, and stability of the PM supplement were performed. Six randomly selected pills were tested for metabolic impurities of the metabolites PMP, PLP, PL and PN by UPLC-MS/MS. Furthermore, purity of the pyridoxamine supplement was tested using NMR analysis. Both a ^{1}H and ^{13}C spectrum of 50 mmol/L pyridoxamine·HCl unbuffered in D_2O were made. Proton and carbon resonance positions were compared in duplo measurements.

Results

Method of B6 quantification

Chromatography and tandem mass spectrometry

B6 vitamers were chromatographically separated and detected with MRM in the positive ESI mode. Optimized mass transitions, cone voltages, collision energies, retention times, and corresponding stable isotope internal standards are shown in Table 6.1.

Table 6.1 Chromatographic and tandem mass spectrometric conditions.

Compound	Precursor ion ($+H^+$)	Product ion	Cone (V)	Collision (eV)	Retention time (min)
PM	169.1	152.2	30	12	3.23
D_3-PM	172.1	155.2	30	12	3.23
PMP*	249.2	232.2	30	16	2.33
PLP	248.15	150.15	20	16	0.52
D_3-PLP	251.15	153.15	20	16	0.52
PL	168.2	150.2	20	9	2.27
D_3-PL	171.2	153.2	20	9	2.27
PN	170.1	152.1	20	17	2.34
D_3-PN	173.1	155.1	20	17	2.34

*Internal standard D_3-PN was used. PM: pyridoxamine, PMP: pyridoxamine 5′-phosphate, PLP: pyridoxal 5′-phosphate, PL: pyridoxal, PN: pyridoxine.

Method validation

Calibration curves for the B6 vitamers were linear over the described concentration ranges in water, urine and plasma matrix (for all; r^2>0.99). Mean slopes (response factors, Rf) for PM, PMP, PLP, PL and PN as tested in different matrices are shown in Table 6.2.

Inter- and intra assay coefficients of variation and recoveries of the B6 vitamers as determined by replicate analysis of a pooled EDTA plasma and urine sample, with and without spike, were between 1.1 and 16% (Table 6.3a and 6.3b). Mean recovery for PM and the other B6 vitamers was ~92%. Lower limit of quantification (LOQ) at a signal-to-noise (s/N) ratio of 10 in plasma was 11.2 nmol/L for PM, 4.6 nmol/L for PMP, 121.4 nmol/L for PLP, 42.1 nmol/L for PL, and 6.3 nmol/L for PN. LOQ in urine was 110.2 nmol/L for PM, 41.1 nmol/L for PMP, 650.8 nmol/L for PLP, 236 nmol/L for PL and 413.5 nmol/L for PN.

Table 6.2 Determination of response factors of B6 vitamers as determined in the matrices water, plasma, and urine.

Matrix	PM	PMP	PLP	PL	PN
Water	0.7349	0.2053	0.3512	0.9829	0.8425
Plasma A	0.7689	0.2012	0.3376	0.9678	0.8281
Urine A	0.7461	0.2848	0.3597	0.9674	0.8028
Plasma B	0.7305	0.3054	0.2632	1.0523	0.9132
Urine B	0.7477	0.3538	0.2485	0.9729	0.7984
Mean (Rf)	0.7456	0.2701	0.3120	0.9887	0.8370
Stdev	0.0149	0.0660	0.0522	0.0361	0.0463
CV%	2.0	24.4	16.7	3.7	5.5

CV: coefficient of variation (%), PM: pyridoxamine, PMP: pyridoxamine 5′-phosphate, PLP: pyridoxal 5′-phosphate, PL: pyridoxal, PN: pyridoxine.

Purity of the pyridoxamine supplement

Six randomly selected pills were tested for PM and metabolite content. We found an average content of 77.3 ± 2.3 mg PM/pill (theoretical PM content: 66.7 mg/pill). Other B6 vitamers (PMP, PLP, PL, and PN) were not detectable. Proton and carbon resonance positions of pyridoxamine.2HCl in duplo measurements gave identical results (Supplementary Figure S6.1), confirming the molecular structure of PM (Supplementary Figure S6.2) and the purity of the supplement.

Table 6.3a Recovery and precision data of B6 vitamers in plasma.

Compound Intra-assay (n=6)	Plasma Mean (SD) nmol/L	CV%	Added nmol/L	Plasma + spike Mean (SD) nmol/L	CV%	Recovery %
PM	1004 (16)	1.6	3741	4725 (54)	1.1	99.5
PMP	ND*	ND*	61	56.8 (0.8)	1.5	92.9
PLP	1013 (6)	0.6	752	1505 (23)	1.5	65.4
PL	2105 (64)	3.1	747	2616 (74)	2.8	68.4
PN	18.9 (1.0)	5.1	747	750 (37)	4.9	97.9

Compound Inter-assay (n=3)	Plasma Mean (SD) nmol/L	CV%	Added nmol/L	Plasma + spike Mean (SD) nmol/L	CV%	Recovery %
PM	962 (26)	2.7	3741	4689 (39)	0.8	99.9
PMP	ND*	ND*	61	59.8 (5.0)	8.3	97.9
PLP	994 (15)	1.5	752	1596 (112)	7.0	80.0
PL	2062 (33)	1.6	747	2802 (175)	6.2	99.0
PN	18.6 (0.9)	4.8	747	777 (23)	3.0	101.5

*ND = not detectable. CV: coefficient of variation (%), PM: pyridoxamine, PMP: pyridoxamine 5′-phosphate, PLP: pyridoxal 5′-phosphate, PL: pyridoxal, PN: pyridoxine

Table 6.3b Recovery and precision data of B6 vitamers in urine.

Compound Intra-assay (n=6)	Urine Mean (SD) nmol/L	CV%	Added nmol/L	Urine + spike Mean (SD) nmol/L	CV%	Recovery %
PM	44090 (365)	0.8	11222	53900 (455)	0.8	87.4
PMP	ND*	ND*	183	222 (8)	3.4	121.1
PLP	ND*	ND*	2257	1562 (9)	0.6	69.2
PL	5007 (129)	2.6	2240	6607 (255)	3.9	71.4
PN	241 (7)	3.1	2240	2233 (78)	3.5	88.9

Compound Inter-assay (n=3)	Urine Mean (SD) nmol/L	CV%	Added nmol/L	Urine + spike Mean (SD) nmol/L	CV%	Recovery %
PM	43486 (606)	1.4	11222	54296 (374)	0.7	96.3
PMP	ND*	ND*	183	230 (18)	7.8	125.2
PLP	ND*	ND*	2257	1755 (281)	16.0	77.7
PL	4416 (341)	7.7	2240	6666 (68)	1.0	100.5
PN	240 (4.1)	1.7	2240	2291 (57)	2.5	91.5

*ND = not detectable. CV: coefficient of variation (%), PM: pyridoxamine, PMP: pyridoxamine 5′-phosphate, PLP: pyridoxal 5′-phosphate, PL: pyridoxal, PN: pyridoxine.

Metabolization of pyridoxamine

Pharmacokinetics of a single dose of 200 mg PM

After a single oral dose of 200 mg PM, PM and its metabolites increased in plasma (Figure 6.2A, B, C, D, and E). PM increased from 1.1 ± 1.1 nmol/L at baseline to a maximum of 2324 ± 266 nmol/L and PMP from 0.1 ± 0.04 to 4.1 ± 1.7 nmol/L within ~3 hours (mean ± SEM). PM and PMP levels in plasma returned to baseline after ~10 hours of PM intake. PM showed a half-life of approximately ~1 hour. PLP increased from a baseline level of 346 ± 53 to a maximum of 2787 ± 329 nmol/L after ~10 hours and reached a plateau. PL increased from a baseline level of 15.0 ± 1.7 to a maximum of 1921 ± 185 nmol/L after ~6 hours. PN was at baseline undetectable and increased within ~6 hours to 13.5 ± 2.1 nmol/L. PL did not completely normalize within 24 hours and PN normalized after ~12 hours. Fasted second void urinary PM, PL, and PN concentrations were 2.3 ± 0.6, 21.2 ± 5.1, and 0.1 ± 0.04 nmol/mmol creatinine, respectively (Figure 6.3A, B, and C) and were increased to 12.3 ± 1.7, 137 ± 30.7, and 0.7 ± 0.3 nmol/mmol creatinine, respectively, after a single-oral dose of 200 mg PM. Twenty-four hour urine concentrations were 5696 ± 738, 1489 ± 349, and 39.8 ± 8.0 nmol/mmol creatinine for PM, PL, and PN, respectively. PMP and PLP were not detectable in urine.

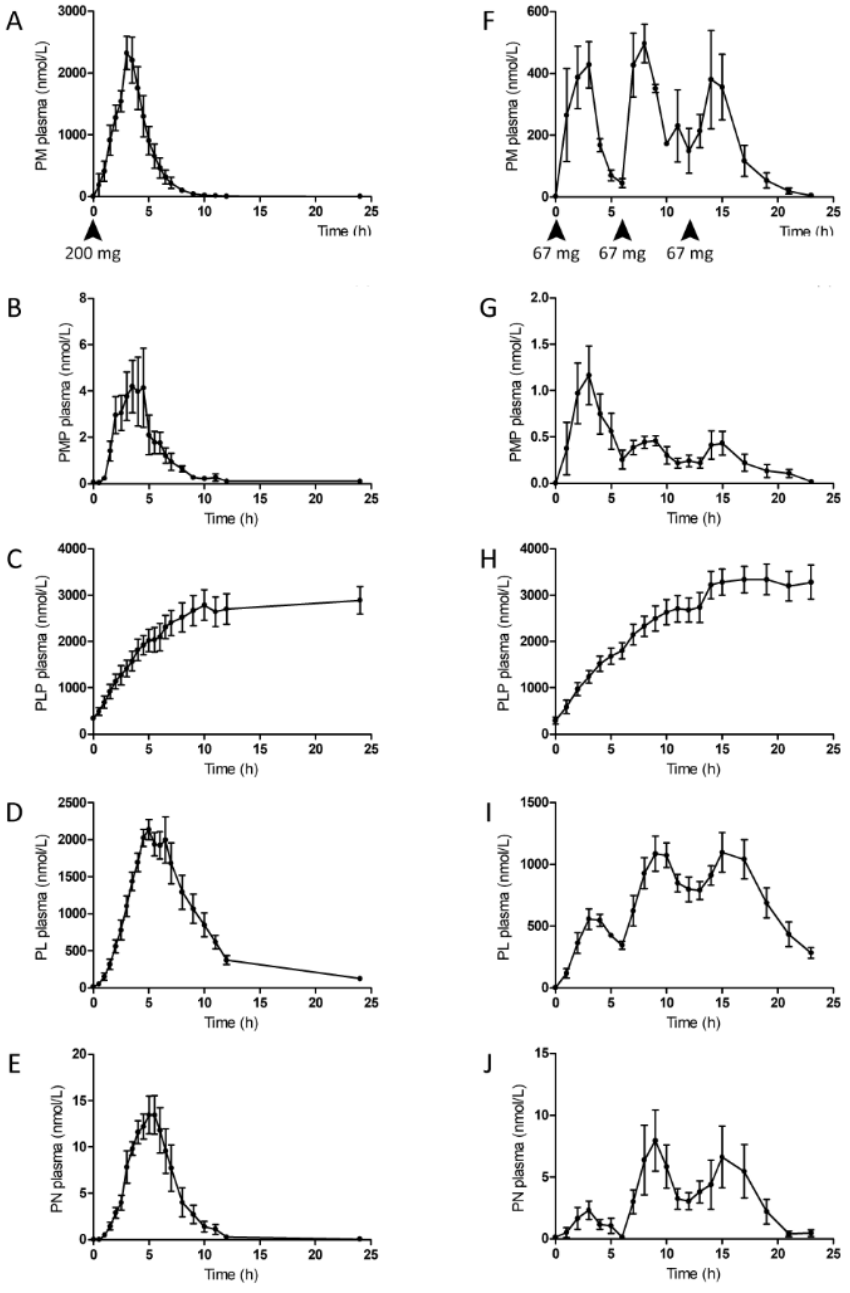

Figure 6.2 Plasma time-concentration plots of PM and its metabolites after a single oral dose of 200 mg PM (A, B, C, D, and E) and a three daily oral dose of 67 mg PM (F, G, H, I, en J). Black arrows: intake PM supplement. PM: pyridoxamine, PMP: pyridoxamine 5′-phosphate, PLP: pyridoxal 5′-phosphate, PL: pyridoxal, PN: pyridoxine, PNP: pyridoxine 5′-phosphate.

Pharmacokinetics of a three daily dose of 67 mg PM

With a three daily oral dose of 67 mg PM every 6 hours, we observed an increase and decline of PM, PMP, PL, and PN concentrations after each PM intake (Figure 6.2F, G, I, and J). PLP showed an accumulation over time and did not decline after 24 hours (Figure 6.2H). Maximum plasma concentrations of PM, PMP, PLP, PL, and PN were 497 ± 63, 1.2 ± 0.3, 3282 ± 281, 1072 ± 99, and 7.9 ± 2.5 nmol/L (mean ± SEM), respectively. Fasted second void urinary PM, PL, and PN concentrations were 2.2 ± 0.5, 24.9 ± 6.1, and 0.2 ± 0.1 nmol/mmol creatinine, respectively (Figure 6.3D, E, and F). After a three daily dose of 67 mg PM, fasted second void urinary PM, PL, and PN concentrations were increased to 51.1 ± 27.0, 239 ± 27.6, and 3.6 ± 1.1 nmol/mmol creatinine, respectively. Twenty-four hour urine concentrations were 3586 ± 416, 1649 ± 180, and 50.5 ± 7.3 nmol/mmol creatinine for PM, PL, and PN, respectively. PMP and PLP were not detectable in urine.

Effect of dietary intake

Dietary intake did not substantially change plasma and urine B6 vitamer concentrations during the day (Supplementary Table S6.1). Maximum plasma concentrations of PM, PMP, PLP, and PL were 3.8 ± 0.6, 0.7 ± 0.3, 401 ± 123, and 11.7 ± 2.4 nmol/L, respectively (Supplementary Figure S6.3). PN was not quantifiable in plasma (<LOQ). Fasted second void urine content was comparable on the day before and after the dietary schedule, and within the expected physiological range; average concentrations of PM, PL, and PN in the second void urine were 2.5 ± 0.4, 13.1 ± 5.1, 0.4 ± 0.3 nmol/mmol creatinine, respectively. Twenty-four hour urine concentrations were 3.4 ± 0.4, 23.4 ± 16.2, and 0.4 ± 0.2 nmol/mmol creatinine for PM, PL, and PN, respectively (Supplementary Figure S6.4).

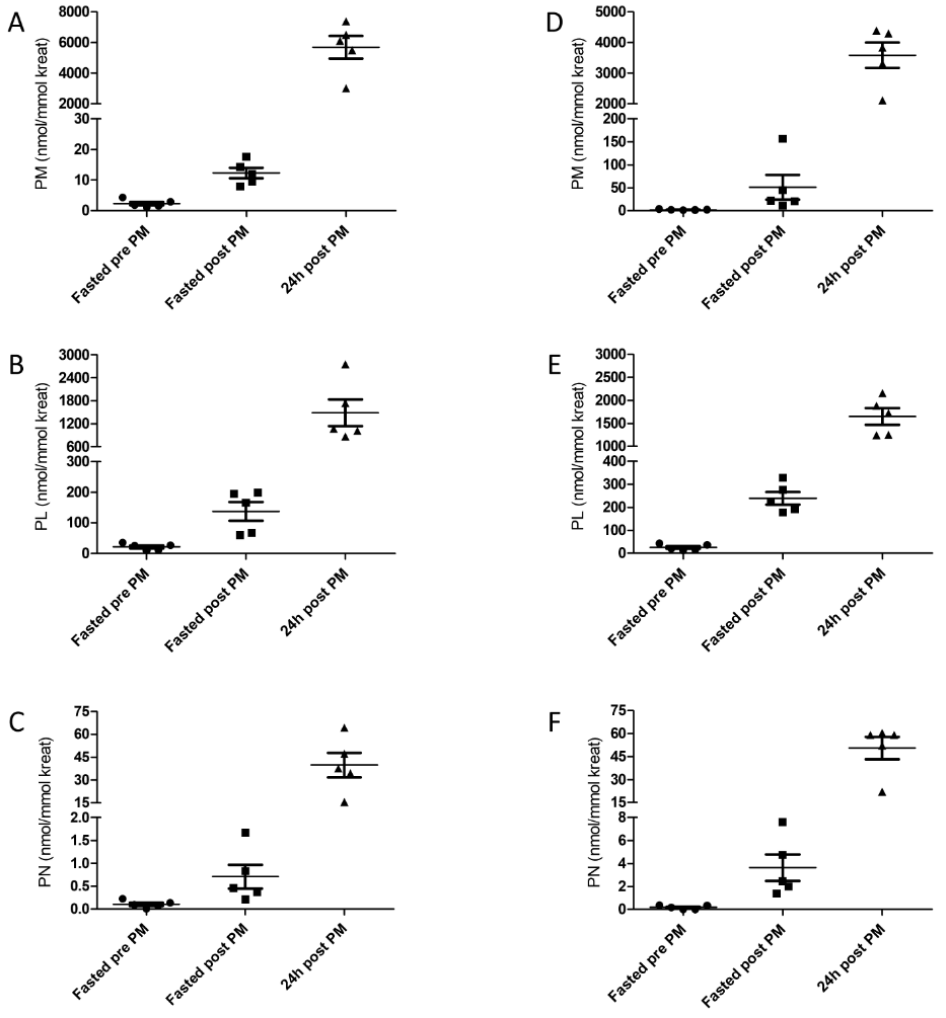

Figure 6.3 Fasted second void urine concentrations of PM, PL, and PN before (dot) and after (square) a single oral dose of 200 mg PM (A, B, and C), and before (dot) and after (square) a three daily oral dose of 67 mg PM (D, E, and F). PM, PL, and PN concentrations in 24-hour urine after a single-oral dose of 200 mg PM (A, B, and C, triangle) and after a three daily oral dose of PM (D, E, and F, triangle). PM: pyridoxamine, PMP: pyridoxamine 5′-phosphate, PLP: pyridoxal 5′-phosphate, PL: pyridoxal, PN: pyridoxine, PNP: pyridoxine 5′-phosphate.

Discussion

In this study we analyzed the pharmacokinetics and metabolization of PM in a substudy of a clinical trial. We first optimized and validated a method for the quantification of vitamin B6 in plasma and urine using UPLC-MS/MS. With this method we found increased levels of PM and the biologically active PLP after PM supplementation, with minimal formation of PN.

PM is a known chemical scavenger of reactive oxygen species and reactive dicarbonyl species such as methylglyoxal (MGO), and has been shown to inhibit the formation of AGEs and ALEs[17,39-42]. Clinical trials in patients with diabetic nephropathy and patients with schizophrenia showed moderate improvements with dosages ranging from 100 mg/day up to 2400 mg/day, with no established adverse effects[21-23]. In addition, after a maximum dose of 11.6 mmol/kg, PM did not cause any clinical signs of neurotoxicity in rats[43]. These studies suggest that PM may target physiological pathways relevant to the progression of metabolic and age-related diseases, without adverse effects.

Although several clinical trials with PM have been performed[21-23], the current study is the first report about of PM metabolization in humans. We showed a rapid uptake and metabolization of the PM supplement. After 30 minutes, plasma concentrations of PM and PLP were increased. This short lag time is likely explained by the fact that B6 vitamers are water-soluble and are absorbed in the gut by non-saturable passive diffusion of the non-phosphorylated forms[44]. In the first three hours we saw an increase in plasma PM. PMP is slightly increased in plasma, and rapidly converted into PLP and PL. Indeed, PL showed a strong increase in the first six hours, and the biologically active PLP showed a plateau phase at its maximum concentration. Both a single dose of 200 mg PM, as well as a three daily dose of 67 mg PM resulted in a steady increase of PM and the biologically active form PLP. The three daily dose resulted in a lower, yet more constant PM concentration during the day. Urine concentrations in both experiment groups showed comparable amounts of PM, PL and PN in the 24 hour urine collection. After a 12 hour overnight fast, very little was still detectable in the second void urine samples. As expected, large portions of these metabolites were excreted via urine.

In both the single dose and three daily dose experiments, we showed maximum plasma PN concentrations below 15 nmol/L. The upper limit of PN intake according to the current EFSA safety regulations is 25 mg/day[45]. Based on the assumption that 25 mg PN would be taken up completely, in an average blood volume of five liter, the maximum PN concentration would be approximately 30 µmol/L. Thus, the conversion of PM to PN in our study is very low. Nevertheless, the formation of PN is remarkable as previous studies presumed that conversion to PN from metabolites other than PNP was not

possible. Since we did not find any impurities in our PM supplements and did not show other B6 vitamers than PM in the supplement, it is most likely that a small portion of the PM supplement is converted to PN. Indeed, a recent study discovered a pyridoxal reductase as part of the human B6 metabolism. This enzyme is responsible for the reduction of PL to PN, and can explain the small amount of PN in our experiment[46]. This possibility is supported by the study of Vrolijk et al., who found a small increase in plasma PN after supplementation with PLP[47].

Thus, only a small amount of PM was metabolized to PN, which is very beneficial with regards to potential toxicity of PN. Although vitamin B6 is an important enzymatic cofactor in a wide range of physiological processes[3], it has become clear there is a delicate balance between biochemical activity, and cellular toxicity with vitamin B6 supplementation. The vitamer PN in particular appears to be the main culprit in vitamin B6 toxicity[34,36,37]. PN is the most widely used and available form of vitamin B6 supplementation. It has been demonstrated in in vitro experiments that PN induces cell death, while the other vitamers including PM did not[37]. Paradoxically, a supplementation with PN can induce similar pathophysiological effects, as vitamin B6 deficiency. This can be explained by the competitive inhibition of active PLP by PN[35,37]. Many clinical studies confirm predominantly neurological side effects of PN, and discourage the use of excessive PN dosages[33,47-52]. Although to a lesser extent, side effects of PLP supplementation have also been reported[53,54].

While both PN and PLP can be vital therapeutics[55], it is clear that treatment is not without health risks. Previous studies encourage the research and application of other B6 vitamers[35,37]. We now showed that PM could be more suitable for supplementation because of the very low formation of PN by PM. Thus, the clinical use of PM could offer several benefits for the treatment of vitamin B6 deficiency, but also for treatment of metabolic diseases in which increased dicarbonyls are involved.

Strengths of this study include the use of gold standard UPLC-MS/MS analyses and the validation of this method to analyze B6 vitamers in plasma and urine. To the best of our knowledge, this is the first time PM supplementation and metabolization has been studied in this straightforward approach. The study was well standardized, making use of a pure PM supplement, two supplementation approaches, and a control experiment using the same standardized diet. This study also has important clinical relevance in relation with clinical trials with pyridoxamine since a good evaluation of the compliance and metabolite profile in such intervention studies is of importance. This study also has several limitations, including the small sample size. However, based on the low intra- and inter-assay variation (<5%) observed for pyridoxamine and its metabolites, we believe that a sample size of five healthy subjects with no apparent metabolic disease is sufficient to present reproducible metabolization curves. Furthermore, we also did not

include pyridoxic acid; this catabolite could provide additional information on the total excreted amount of vitamin B6.

In summary, we showed high plasma levels of PM after oral PM supplementation. The three daily dose resulted in a more constant plasma concentration during the day. We found steadily increasing levels of the biologically active PLP after PM supplementation, with minimal formation of PN. Thus, the B6 vitamer PM could be an interesting supplement with regards to the treatment of metabolic diseases and vitamin B6 deficiency.

Acknowledgements

The authors of the present study declare no conflict of interest.

Mathias Van den Eynde and Jean Scheijen performed all experiments. Mathias Van den Eynde, Jean Scheijen and Casper Schalkwijk designed the study and wrote the manuscript. Toshio Miyata provided essential materials. Toshio Miyata and Coen Stehouwer provided a critical review of the manuscript. Casper Schalkwijk supervised the study. All authors read and approved the final manuscript.

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Supplemental materials

Table S6.1 Dietary intake during test days.

Breakfast
22 g Cereals (Granola)
220 mL Yogurt
1 Slice of whole wheat bread with 1 slice of cheese
250 mL Orange juice
250 mL Cappuccino
In-between-snack
1 Slice of whole wheat bread with chocolate sprinkles
250 mL Cappuccino
Lunch
1 Slice of whole wheat bread with 1 slice of cheese
1 Slice of white bread with Sandwich spread
1 Slice of whole wheat bread with marmalade
250 mL Orange juice
250 mL Milk semi-skimmed
1 Tangerine
Diner
250 g Lasagna (vegetarian)
1 Small pizza (vegetarian)
250 mL Ice tea
200 mL Vanilla custard with cookies

Ad libitum water intake during day and night.

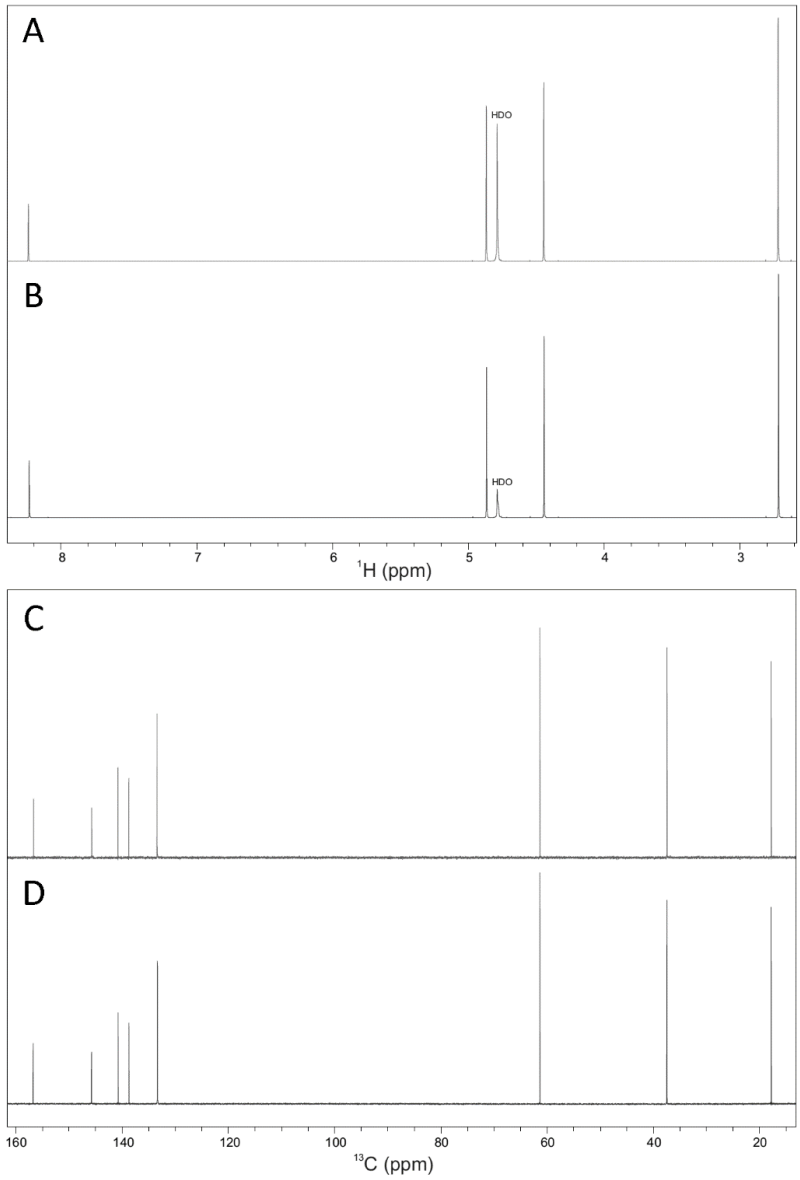

Figure S6.1 1H NMR spectra (A,B) and 13C NMR (C,D) spectra of pyridoxamine.2HCl. Proton and carbon resonance positions were measured and compared in duplo with identical results.

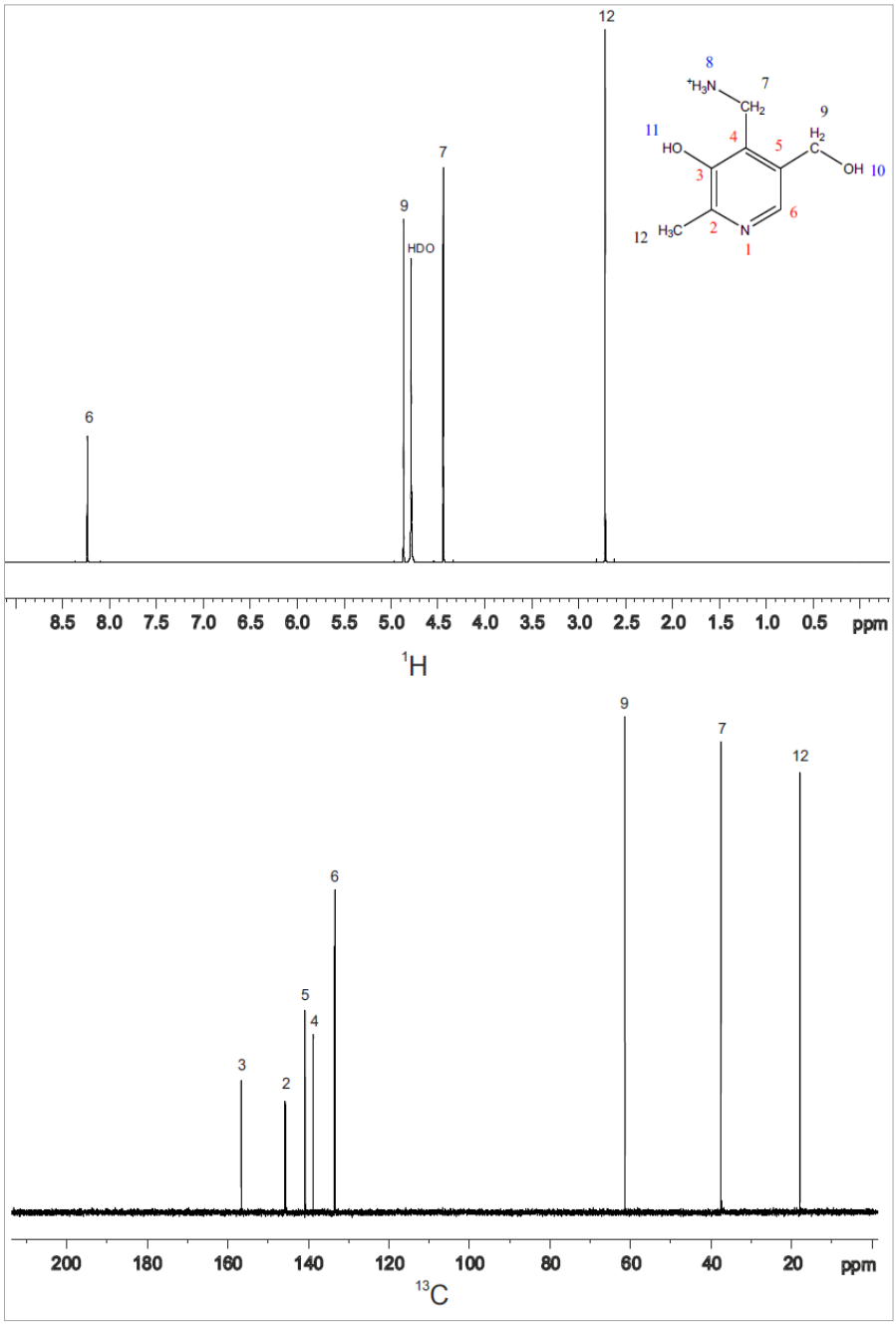

Figure S6.2 1H NMR and 13C NMR spectrum of pyridoxamine-2HCl, with specified molecular structure of pyridoxamine and its peak assignments.

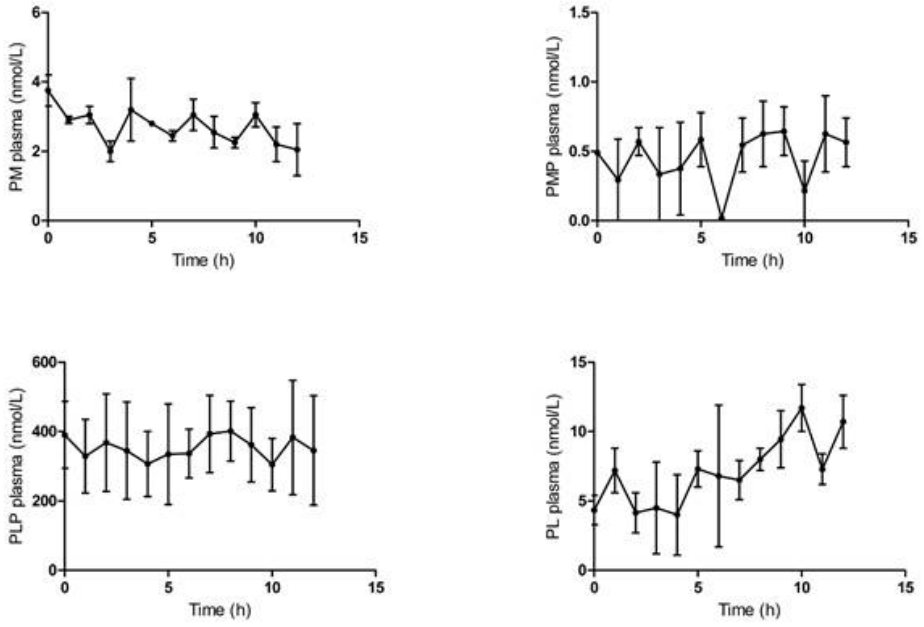

Figure S6.3 Plasma concentration (nmol/L) of PM, PMP, PLP and PL over time (h) during the dietary schedule without PM supplementation (protocol 3). PM: pyridoxamine, PMP: pyridoxamine 5′-phosphate, PLP: pyridoxal 5′-phosphate, PL: pyridoxal.

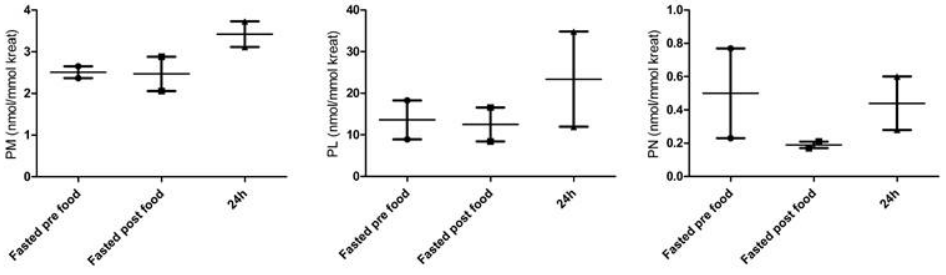

Figure S6.4 Fasted second void urine concentrations (nmol/mmol creatinine) of PM, PL, and PN before (dot) and 24 hours after (square) following the dietary schedule without PM supplementation (protocol 3). PM, PL, and PN concentrations in the complete 24-hour urine collection (triangle) after the dietary schedule without PM supplementation (protocol 3). PM: pyridoxamine, PL: pyridoxal, PN: pyridoxine.

Chapter 7

Pyridoxamine reduces methylglyoxal and markers of glycation and endothelial dysfunction, but does not improve insulin sensitivity or vascular function in abdominally obese individuals: a randomized double-blind placebo-controlled trial

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Abstract

Aims

Dicarbonyl compounds and advanced glycation endproducts (AGEs) contribute to the metabolic and vascular complications of obesity. Pyridoxamine (PM), a B6 vitamer and dicarbonyl scavenger, has been shown to inhibit AGE formation and inflammation, and to improve vascular function and insulin sensitivity in preclinical studies. Therefore, we investigated the effects of PM on glycation, and a large panel of metabolic and vascular measurements in an RCT in abdominally obese individuals.

Materials and methods

Individuals (54% female; mean age 50yr; mean BMI 32kg/m^2) were randomized to an eight-week intervention with either placebo (n=36), 25mg PM (n=36), or 200mg PM (n=36). We assessed insulin sensitivity, β-cell function, insulin-mediated microvascular recruitment, skin microvascular function, flow-mediated dilation, and plasma inflammation and endothelial function markers. PM metabolites, dicarbonyls, and AGEs were measured by UPLC-MS/MS. Treatment effects were evaluated by one-way ANCOVA.

Results

In the high PM dose group, we found a reduction of plasma methylglyoxal and protein-bound Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine, as compared to placebo. We found a reduction of the endothelial dysfunction marker sVCAM-1 in the low and high PM dose group and of sICAM-1 in the high PM dose, as compared to placebo. We found no treatment effects on insulin sensitivity, vascular function, or other functional outcome measurements.

Conclusions

This study shows that PM is metabolically active and reduces MGO, AGEs, sVCAM-1, and sICAM-1, but does not affect insulin sensitivity and vascular function in abdominally obese individuals. The reduction of the adhesion markers is promising since these are important in the pathogenesis of endothelial damage and atherosclerosis.

Introduction

People with abdominal obesity have a high risk of developing insulin resistance and vascular dysfunction[1]. Accumulation of dicarbonyl compounds and advanced glycation endproducts (AGEs) in obesity may be one of the drivers of insulin resistance and vascular dysfunction, ultimately leading to (pre-)diabetes and cardiovascular disease (CVD)[2].
Dicarbonyl compounds such as methylglyoxal (MGO) are reactive metabolites, mainly formed as a byproduct of glycolysis and lipid-oxidation. Dicarbonyls react with proteins to form AGEs, with MGO being the major precursor in the formation of AGEs. We previously showed that dicarbonyls are elevated in abdominally obese individuals[3,4]. MGO and MGO-derived AGEs are linked to vascular dysfunction and adipose tissue inflammation[5-9] and have been associated with the development of many age-related diseases including hypertension[10], atherosclerosis[11], and incident cardiovascular disease in both type 1 and type 2 diabetes[12,13].

Pyridoxamine (PM), a B6 vitamer and a chemical scavenger of dicarbonyls, has been shown to inhibit AGE formation[14-17]. Several preclinical studies have demonstrated that PM is efficacious in the prevention of nephropathy[18-22], the improvement of lipidemia, proteinuria, and atherosclerosis[23]. In humans, large-scale clinical trials with PM showed a moderate improvement of eGFR in patients with overt nephropathy and in individuals with less severe renal impairment[24,25].
In obese mice, we previously showed that PM improves glucose tolerance and insulin metabolism, and prevents adipose tissue inflammation and vascular dysfunction[7]. Additionally, others have shown that PM decreased fasting insulin levels and improved insulin sensitivity in obese and type 2 diabetic mice[26,27], most likely by trapping MGO and inhibiting AGE formation[28,29].

So far, no clinical studies have investigated the potential of PM in the prevention of metabolic and vascular dysfunction by lowering MGO in the setting of obesity. Therefore, we investigated the effects of eight weeks of PM supplementation on glycation, insulin sensitivity, and micro- and macrovascular function in a randomized placebo-controlled trial in abdominally obese individuals.

Materials and methods

Study population and design

We studied individuals with abdominal obesity, defined as a waist circumference above 102 cm for men and above 88 cm for women. Individuals were non-diabetic; both individuals with a normal and impaired glucose tolerance participated in the study. Individuals with current metabolic or vascular disease were excluded. All participants gave written informed consent before enrolment in the study. The study was carried out in accordance with the Declaration of Helsinki and was registered at the ClinicalTrials.gov database with identifier number NCT02954588. All study measurements took place at Maastricht University Medical Center (MUMC+) in the Netherlands.
A total of five visits were scheduled: a screening visit, two experimental test days before, and two identical test days after the PM intervention. After baseline measurements, participants were randomized to an eight-week intervention with either placebo treatment, 25 mg/day PM (low dosage), or 200 mg/day PM (high dosage), with a randomization ratio of 1:1:1. The randomization was stratified for sex and age. A full description of the methods is provided in the supplement.

Pyridoxamine supplementation and compliance

The high dose group took a total daily dose of 200 mg PM, the low dose group a total daily dose of 25 mg PM. To ensure a steady plasma concentration during the day, the daily dosage was supplied as three capsules per day. The placebo group consumed three capsules of potato starch. To evaluate compliance, remaining capsules were counted after study completion. PM metabolites (individual PM vitamers) were measured in fasting plasma and 24h urine samples of the last study day, these data were compared to our previous metabolization study with the same PM supplement[30].

Dietary intake and physical activity

During the intervention period, individuals were asked not to change their lifestyle, diet, and physical activity routines, and to avoid excessive alcohol consumption or the use of dietary supplements. Individuals received clear instructions and possible illness or interfering events were documented. A validated food frequency questionnaire (FFQ)[31] was completed to evaluate habitual diet and average AGE intake, based on dietary AGE concentrations previously determined in a large food database[32]. Prior to the test days, individuals adhered to an overnight fast and several standardization procedures.

Oral glucose tolerance test and hyperinsulinemic, euglycemic clamp

Glucose tolerance was assessed with a two-hour seven-sample oral glucose tolerance test (OGTT). The glucose incremental area under the curve (iAUC), the insulin iAUC and c-peptide iAUC were measured. β-cell function was assessed by calculating early-phase (first 30 minutes) and late phase indices (after 120 minutes) using the OGTT data.
Metabolic insulin sensitivity was determined with the euglycemic, hyperinsulinemic clamp method, as previously described[33,34]. Whole body glucose uptake was calculated from the infusion rate during the last hour of the clamp (90-150 min) and expressed per kg body weight (GIR) and expressed per unit of plasma insulin concentration measured in this time interval (M/I-value).

Vascular measurements

As a measure of microvascular insulin sensitivity, insulin-mediated muscle microvascular recruitment (IMMR) was assessed by means of contrast-enhanced ultrasound (CEUS), before and during hyperinsulinemia, as previously described[35]. IMMR was calculated as the relative increase (%) in muscle microvascular blood volume during hyperinsulinemia.
We measured flowmotion of skin microcirculation by means of laser Doppler flowmetry on the skin of the left forearm, as described earlier[36]. In addition, we evaluated skin heat-induced hyperemic, endothelium-dependent, vasodilation with a second laser Doppler probe, under local heating to 44°C, as described previously[37].
Brachial artery flow-mediated vasodilation (FMD) was measured using continuous ultrasound echography as previously described[38]. The FMD response was quantified as the change in brachial artery diameter relative to reference diameter (pre-occlusion), and additionally normalized to stimulus (i.e. the relative increase in blood flow velocity with hyperemia).
The endothelial glycocalyx thickness was determined using the GlycoScan device (GlycoCheck, the Netherlands).

Dicarbonyls and AGE measurements in plasma and urine

Levels of dicarbonyls and AGEs were measured in EDTA plasma samples. Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to determine the plasma levels of methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG), as previously described[39,40].
Skin Autofluorescence (SAF), an estimate of tissue AGEs, was measured with the AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands), as described in previous publications[41,42].

Biomarkers, liver and kidney function

Plasma biomarkers of endothelial dysfunction, low-grade inflammation, insulin, and c-peptide were assessed using a validated and commercially available multi-array detection system, based on electro-chemiluminicence technology and ELISA.
Creatinine, Gamma-GT, total cholesterol, HDL-cholesterol, triglycerides, HbA1c, and fasting-insulin were determined in serum samples at the Central Diagnostic Laboratory of the MUMC+. LDL-cholesterol was calculated using the Friedewald formula[43]. Plasma glucose levels during the oral glucose tolerance test and insulin clamp were determined with a YSI2300 glucose analyzer (YSI, Yellow Springs, Ohio, USA).
The fatty liver index (FLI) was calculated using waist circumference, BMI, plasma triglyceride, and plasma gamma-glutamyl-transferase (GGT)[44]. Kidney function was assessed using the CKD-EPI creatinine equation for the estimation of the glomerular filtration rate (eGFR)[45].
In whole blood samples we performed an immune cell differentiation test measuring the amount of circulating leucocytes, segmented granulocytes, lymphocytes, monocytes, eosinophils, and basophils.

Statistical analysis

We evaluated two main outcomes (insulin sensitivity and microvascular function) in our study. PM metabolites served as a measure of compliance. MGO was assessed to evaluate the effectiveness of the intervention. The other outcome parameters were exploratory.
Normally distributed variables are presented as means ± standard deviations, or as medians with interquartile ranges when variable distribution was skewed. Non-normally distributed values were log transformed prior to analysis. Treatment effects in the high and low dose intervention groups were assessed in comparison to the placebo group and were analyzed by means of one-way ANCOVA with adjustment for baseline values. Differences between the study groups in repeated measures during the OGTT were analyzed using mixed ANOVA with intervention as between-subjects factor and time within-subjects factor. For the mixed ANOVA intervention analysis, the baseline postprandial curve was subtracted from the postprandial curve at follow-up. A Jonckheere-Terpstra test was used to assess a trend in median values over the three study groups. Regression analysis was used to test the relation between pyridoxamine metabolites and plasma markers at follow-up. We corrected the treatment analysis for age and sex to evaluate possible confounding effects. As no changes in outcome after this correction were observed, the final data presented are adjusted solely for baseline levels, and compared pairwise to the placebo intervention. Presented treatment effects

in the figures and tables are weighted means corrected for baseline values and in comparison to the placebo group. A Z-score was calculated for all glycation markers combined, as the average Z-score of individual Z-scores of nine plasma glycation markers. P values ≤0.05 were considered statistically significant. All statistical analyses were performed with IBM SPSS Statistics version 25.

Results

Trial characteristics

A CONSORT flow diagram of participant inclusion throughout the RCT is shown in Supplementary Figure S7.1. At the end of the study, 36 individuals per group had finished all primary outcome measurements at the follow-up visit. Age, sex, anthropometric values and metabolic markers were comparable at baseline between all three groups (Table 7.1).

Table 7.1 Study population characteristics at baseline.

	Placebo (n=36)	Pyridoxamine 25 mg/day (n=36)	Pyridoxamine 200mg/day (n=36)
Age [Yr]	48.9±12.8	50.7±13.8	50.6±12.5
Sex [%female]	55.6	52.8	52.8
Weight [kg]	97.1±17.7	95.1±14.7	94.5±20.3
Fat percentage by skinfold	38.1±5.9	38.1±4.9	37.8±5.8
BMI [kg/m^2]	32.2±5.9	32.0±4.0	31.8±5.1
Waist Circumference [cm]			
Male	113±12	113±9	112±12
Female	107±13	100±8	102+13
Waist to hip ratio	0.98±0.1	0.96±0.1	0.98±0.1
Fasting Plasma Glucose [mmol/L]	5.1±0.5	5.1±0.5	5.1±0.3
Fasting Plasma Insulin [mIU/L]	16.6±9.8	14.2±7.5	15.7±10.5
Two-hour Plasma Glucose [mmol/L]	7.6±1.8	7.7±2.0	7.2±1.6
HbA1c [%]	5.3±0.4	5.3±0.4	5.3±0.4
Total Cholesterol [mmol/L]	4.9±0.8	4.7±0.6	5.0±1.0
LDL Cholesterol [mmol/L]	1.2±0.3	1.2±0.4	1.2±0.3
HDL Cholesterol [mmol/L]	3.4±0.8	3.2±0.6	3.5±1.0
Triacylglycerol [mmol/L]	1.2[0.9-1.7]	1.3[0.9-1.5]	1.4[1.1-1.7]
24-h Systolic BP [mmHg]	123±11	123±11	124±12
24-h Diastolic BP [mmHg]	78±8	79±8	78±11

Data are presented as mean ± SD, or median [interquartile range] as appropriate, *n*=108 (36 per group). BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; BP, blood pressure. Two-hour glucose as measured during oral glucose tolerance test (OGTT).

Metabolization of PM and compliance

The mean compliance during the intervention was >80% based on the number of returned capsules. High compliance was confirmed based on pyridoxamine metabolites (Supplementary Table S7.1 and Figure S7.2). PM was metabolized rapidly, with a half-life of approximately one hour, as was previously shown[30]. PM is metabolized into pyridoxamine-phosphate (PMP), pyridoxal (PL), and finally pyridoxal-phosphate (PLP). Both plasma pyridoxal and pyridoxal-phosphate concentrations were significantly increased after PM supplementation ($p<0.0001$), with a dose-dependent trend ($p<0.01$) (Supplementary Table S7.1 and Figure S7.2). After eight weeks, pyridoxal-phosphate concentrations were increased by 854 nmol/L in the low dosage group (95%CI [610, 1098]; $p<0.0001$) and by 1676 nmol/L in the high dosage group (95%CI [1435, 1917]; $p<0.0001$), as compared to placebo. Pyridoxal concentrations were unchanged in the low dosage group (+57 nmol/L; 95%CI [-55, 169]; $p=0.32$), but were significantly increased in the high dosage group (+273 nmol/L; 95%CI [165, 380]; $p<0.0001$) as compared to placebo. Pyridoxine (PN) plasma concentrations after eight weeks of the high dose were very low (<2 nmol/L) and showed a non-significant increase after eight weeks of PM treatment.

General anthropometric and metabolic risk markers

Anthropometric measurements were comparable between the three study groups at the start of the study and did not change after eight weeks of intervention. Weight, BMI, fat percentage and waist circumference were not significantly changed due to pyridoxamine supplementation (Supplementary Figure S7.5). Furthermore, fatty liver index (FLI), eGFR, and mean arterial pressure also did not change after intervention (Supplementary Table S7.2). No side-effects of the pyridoxamine intervention were reported.

Dicarbonyl compounds and glycation markers

We found a treatment effect of pyridoxamine on plasma MGO levels between the three study groups ($p=0.036$) with a significant reduction of plasma MGO of 22 nmol/L in the high dosage group compared to the placebo (95%CI [-39, -4]; $p=0.017$) (Table 7.2, Supplementary Figure S7.3). The low dose of pyridoxamine did not significantly reduce MGO levels (-3 nmol/L; 95%CI [-21, 14]; $p=0.7$). A Jonckheere-Terpstra trend test showed that there was a statistically significant trend of lower median MGO concentration post-intervention over the three study groups ($p=0.026$). Furthermore, we found a treatment effect on the major MGO modification, protein-bound MG-H1 ($p=0.016$). The high dose of pyridoxamine decreased protein-bound MG-H1 in plasma with 211 nmol/L (95%CI [-371, -52]; $p=0.010$). This effect was not significant in the low

dose group. The dicarbonyls GO and 3-DG, protein-bound CML, protein-bound CEL, and the free plasma AGEs did not change significantly by PM in comparison to the placebo group. However, a Z-score of all plasma AGEs combined showed a borderline significant decrease of AGEs by PM treatment (Z-score: -0.38; 95%CI [-0.77, 0.02]; p=0.06) (Table 7.2). Tissue AGEs as measured with SAF did not change during the intervention (Table 7.2). Data from food frequency questionnaires (FFQs) demonstrated no apparent differences in dietary AGE intake and dietary dicarbonyl intake between the study groups at baseline (Supplementary Table S7.4). Furthermore, urinary AGEs in twenty-four hour urine collections, which also reflect dietary AGE intake, were unchanged after the intervention (Supplementary Figure S7.4 - C).

Insulin sensitivity, glucose metabolism and β-cell function

Fasting plasma glucose, insulin, and c-peptide concentrations were not significantly different between the three study groups after eight weeks of treatment (Table 7.3).
Measurements of metabolic insulin sensitivity, as determined with the hyperinsulinemic euglycemic clamp, were not statistically different after the intervention; we found no treatment effect on whole body glucose disposal (glucose infusion rate) or the M/I value (Table 7.3).
The glucose incremental area under the curve (iAUC), the insulin iAUC and c-peptide iAUC, all measured during the OGTT, were not affected by pyridoxamine (Supplementary Figure S7.6 and S7.7).
None of the β-cell indices were significantly altered by treatment; we found no changes in C-peptidogenic index, insulinogenic index and corrected insulin response (CIR) after 30 and 120 minutes (Table 7.3).

Micro- and macrovascular function

Insulin-mediated microvascular recruitment (IMMR) in skeletal muscle was not statistically different after PM intervention with a mean increase after the high dose PM treatment of 24% (95%CI [-0.14, 0.61], p=0.21) (Table 7.3). Likewise, skin microvascular flowmotion, skin hyperemic response after local heating, and the sublingual endothelial glycocalyx measurements, were not statistically different between the placebo and low and high dose PM groups (Table 7.3). Finally, brachial artery flow-mediated dilation (FMD) did not show any significant treatment effect (Table 7.3).

Table 7.2 Treatment effects of 8 weeks pyridoxamine on dicarbonyls and AGEs.

	Crude data						Treatment effect			
	Placebo BL Mean±SD	Placebo FU Mean±SD	Low BL Mean±SD	Low FU Mean±SD	High BL Mean±SD	High FU Mean±SD	PM low dose - placebo Mean change (95% CI)	p *value*	PM high dose - placebo Mean change (95% CI)	p *value*
Plasma dicarbonyls										
MGO (nmol/L)	248±45	250±43	251±46	247±34	249±40	228±39	-3 (-21, 14)	0.71	-22 (-39, -4)	0.02
GO (nmol/L)	1590±498	1423±330	1661±426	1539±385	1688±461	1654±489	95 (-87, 277)	0.30	113 (-67, 293)	0.22
3-DG (nmol/L)	1230±134	1234±154	1228±148	1220±121	1201±130	1190±110	-15 (-50, 20)	0.41	-23 (-58, 11)	0.18
Free plasma AGEs										
CML (nmol/L)	98±38	97±45	99±32	99±38	89±33	83±25	0.5 (-14, 15)	0.95	-10 (-24, 5)	0.19
CEL (nmol/L)	60±36	58±27	59±27	59±24	52±20	49±19	1.1 (-9, 11)	0.83	-6 (-17, 4)	0.21
MG-H1 (nmol/L)	167±141	181±145	177±98	152±89	129±70	133±112	-32 (-86, 21)	0.23	-38 (-91, 16)	0.16
Protein-bound plasma AGEs										
Protein-bound CML (nmol/L)	3795±914	3644±765	3772±741	3769±727	3654±777	3497±697	160 (-74, 395)	0.18	-45 (-277, 187)	0.70
Protein-bound CEL (nmol/L)	1142±366	1179±369	1176±265	1134±286	1233±432	1159±382	-57 (-187, 74)	0.39	-72 (-202, 57)	0.27
Protein-bound MG-H1 (nmol/L)	1525±511	1519±494	1489±413	1475±417	1574±641	1386±653	-17 (-179, 145)	0.84	-211 (-371, -52)	0.01
Combined z-score of plasma AGEs	0.06±1.13	0.18±1.14	0.08±0.93	0.13±0.93	-0.14±0.94	-0.30±0.87	-0.07 (-0.47, 0.34)	0.75	-0.38 (-0.77, 0.02)	0.063
Skin autofluorescence (AU)	1.98±0.36	2.03±0.37	2.12±0.34	2.11±0.44	1.91±0.30	1.92±0.37	-0.03 (-0.18, 0.11)	0.65	-0.06 (-0.21, 0.08)	0.37

Treatment effects of pyridoxamine compared to placebo were tested. Treatment effect and mean differences were obtained using one-way ANCOVA with adjustment for baseline values. Unadjusted (crude) data are presented. For the treatment effects, adjusted mean changes (estimated marginal means) with 95% CI are given. All P values are obtained from the ANCOVA analysis with adjustment for baseline values.

Table 7.3 Treatment effects of 8 weeks pyridoxamine on insulin sensitivity, glucose tolerance, β-cell indices, and (micro)vascular function

	Crude data						Treatment effect			
	Placebo BL Mean±SD	Placebo FU Mean±SD	Low BL Mean±SD	Low FU Mean±SD	High BL Mean±SD	High FU Mean±SD	PM low dose - placebo Mean change (95% CI)	P *value*	PM high dose - placebo Mean change (95% CI)	p *value*
Hyperinsulinemic clamp										
M/I (90-150 min)	3.69±2.16	4.03±2.63	4.03±2.17	4.16±2.35	3.75±1.84	4.02±2.24	-0.21 (-0.84, 0.43)	0.52	-0.1 (-0.72, 0.53)	0.76
Whole body glucose disposal (GIR; $mg \cdot kg^{-1} \cdot min^{-1}$)	3.99±1.77	3.95±1.82	4.16±1.85	4.25±2.01	3.88±1.52	3.97±1.65	0.16 (-0.35, 0.66)	0.54	0.08 (-0.42, 0.58)	0.74
Oral glucose tolerance test										
Glucose iAUC (mmol/L x min)	332±162	344±181	346±176	345±198	324±120	321±133	-15 (-64, 33)	0.53	-19 (-67, 28)	0.43
Fasting glucose (mmol/L)	5.11±0.46	5.11±0.49	5.14±0.46	5.10±0.44	5.03±0.33	5.02±0.41	-0.04 (-0.17, 0.1)	0.57	-0.02 (-0.15, 0.12)	0.82
Two-hour glucose (mmol/L)	7.55±1.84	7.48±2.03	7.65±2.04	7.68±2.23	7.17±1.59	7.59±1.57	0.12 (-0.54, 0.79)	0.71	0.37 (-0.29, 1.0)	0.27
HOMA-IR	1.67±0.81	1.47±0.72	1.44±0.62	1.41±0.70	1.44±0.56	1.26±0.50	0.14 (-0.04, 0.3)	0.12	-0.003 (-0.2, 0.2)	0.97
β-cell indices										
C-peptidogenic index (t30)	574±407	478±638	564±448	433±603	476±305	451±318	-28 (-237, 181)	0.79	63 (-145, 271)	0.55
Insulinogenic index (t30)	199±136	173±221	196±159	165±210	175±143	175±161	0.3 (-71, 71)	0.99	30 (-40, 101)	0.40
CIR_{30} ($\times 10^{-2}$)	18±11	15±10	18±15	15±11	17±11	17±12	0.13 (-3.6, 3.9)	0.95	3.3 (-0.38, 7)	0.08
C-peptidogenic index (t120)	2175±8828	824±2823	5±4450	152±7337	181±4871	941±773	-753 (-2924, 1418)	0.49	42 (-2096, 2182)	0.97
Insulinogenic index (t120)	376±1148	231±481	-11±886	184±1494	89±795	205±169	-99 (-531, 333)	0.65	-64 (-488, 359)	0.76
CIR_{120} ($\times 10^{-2}$)	31±28	28±21	30±31	31±34	17±31	23±17	3.9 (-6.7, 14.5)	0.47	1 (-9.6, 11.7)	0.85
(Micro)vascular function										
IMMR (%)	20±45	21±41	19±43	25±58	15±42	44±57	4.9 (-32, 42)	0.79	24 (-14, 61)	0.21
FMD (%)	4.27±4.12	3.53±3.12	3.50±2.18	3.19±2.15	4.13±2.47	2.75±2.49	-0.05 (-1.23, 1.13)	0.93	-0.7 (-1.86, 0.47)	0.24
Skin hyperaemic response (%)	1292±661	1258±553	1268±538	1376±828	1366±703	1363±725	90 (-235, 416)	0.58	84 (-239, 407)	0.61
Endothelial flowmotion (%)	54.62±10.45	55.32±13.60	57.70±11.25	53.83±10.20	56,76±14.45	53.13±11.43	1.9 (-4.7, 8.6)	0.57	-0.3 (-6.9, 6.3)	0.93
Glycocalyx thickness (µm)	2.34±0.22	2.39±0.24	2.38±0.28	2.32±0.23	2.31±0.23	2.41±0.29	-0.09 (-0.2, 0.03)	0.15	0.03 (-0.09, 0.14)	0.66

Treatment effects of pyridoxamine compared to placebo were tested. Treatment effect and mean differences were obtained using one-way ANCOVA with adjustment for baseline values. Unadjusted (crude) data are presented. For the treatment effects, adjusted mean changes (estimated marginal means) with 95% CI are given. All P values are obtained from the ANCOVA analysis with adjustment for baseline values. M/I: Whole-body glucose disposal (M) per unit of plasma insulin concentration (I). Glucose iAUC: The glucose incremental area under the curve (iAUC) was calculated using the trapezoidal rule. HOMA-IR: HOMA2 insulin resistance was based on plasma C-peptide concentrations. IMMR: Insulin mediated microvascular recruitment. FMD: flow-mediated dilation of brachial artery. Skin hyperemic response: this response was measured as the relative increase in skin microvascular flow during local heating. Endothelial flowmotion: the endothelial frequency interval of the skin microvascular flowmotion measurement is reported as a percentage to total flowmotion. Glycocalyx thickness: assessed as perfused boundary region (PBR) and expressed in nanometers.

Plasma biomarkers of endothelial dysfunction

We found an overall treatment effect of pyridoxamine on sVCAM-1 over the three groups (p=0.018) (Table 7.4). We found a reduction in sVCAM-1 of 18 ng/mL in the low dose group (95%CI [-34, -2]; p=0.026) and of 22 ng/mL in the high dose group (95%CI [-38, -6]; p=0.008) as compared to the placebo group. For sICAM-1 we only found a significant reduction in the high dose group of 18 ng/mL (95%CI [-37, -0.1]; p=0.049) (Table 7.4, Figure 7.1). Furthermore, both sICAM-1 and sVCAM-1 showed a significant correlation with plasma concentrations of PLP after the intervention (Figure 7.1). Plasma sE-selectin and vWF did not change in comparison to the placebo group.

Markers of low-grade inflammation and immune cell count

Plasma markers of low-grade inflammation IL-6, IL-8, IL-10, TNF-A, CRP, SAA, and MCP1, did not change after eight weeks of PM intervention (Table 7.4). We also measured circulating leucocytes, segmented granulocytes, lymphocytes, monocytes, eosinophils, and basophils in whole blood; cell counts relative to total leucocytes and absolute counts were not significantly different after eight weeks of pyridoxamine (Supplementary Table S7.3).

Table 7.4 Treatment effects of 8 weeks pyridoxamine on markers of inflammation and endothelial dysfunction

	Crude data						Treatment effect			
	Placebo BL Mean±SD	Placebo FU Mean±SD	Low BL Mean±SD	Low FU Mean±SD	High BL Mean±SD	High FU Mean±SD	PM low dose - placebo Mean change (95% CI)	p *value*	PM high dose - placebo Mean change (95% CI)	p *value*
TNF-α (pg/mL)	1.05±0.26	1.09±0.29	1.07±0.23	1.06±0.19	0.98±0.26	0.99±0.20	-0.04 (-0.11, 0.04)	0.35	-0.05 (-0.13, 0.03)	0.19
IL-6 (pg/mL)	1.01±0.96	0.93±0.63	0.86±0.40	0.91±0.67	0.97±0.79	0.90±0.81	0.045 (-0.26, 0.35)	0.77	-0.01 (-0.31, 0.29)	0.96
IL-8 (pg/mL)	4.02±1.95	4.52±2.25	3.80±0.97	3.74±0.96	3.86±0.94	4.25±2.53	-0.54 (-1.53, 0.46)	0.29	-0.10 (-1.1, 0.89)	0.84
IL-10 (pg/mL)	0.32±0.11	0.33±0.16	0.43±0.40	0.39±0.21	0.32±0.13	0.32±0.12	0.02 (-0.05, 0.09)	0.51	-0.01 (-0.08, 0.06)	0.79
CRP (µg/mL)	3.62±4.90	4.19±5.55	4.61±5.73	5.19±7.10	3.39±4.64	3.38±4.80	0.62 (-1.6, 2.9)	0.58	-0.6 (-2.8, 1.6)	0.59
MCP-1 (µg/mL)	108.84±20.27	118.22±24.06	104.92±24.75	111.62±24.73	108.76±23.30	115.47±22.54	-3.8 (-10.7, 3.2)	0.29	-2.7 (-9.5, 4.2)	0.44
SAA (µg/mL)	5.70±4.91	6.40±6.77	5.85±7.48	7.46±9.73	4.69±4.53	5.32±5.40	1.06 (-2.3, 4.4)	0.53	-0.67 (-4, 2.6)	0.69
Leptin (ng/mL)	18.12±18.70	20.37±19.76	17.17±16.40	18.45±14.91	17.93±18.74	20.40±22.38	-0.04 (-3.9, 3.8)	0.98	0.69 (-3.1, 4.5)	0.72
Adiponectin (ng/mL)	13.85±5.94	14.61±6.21	13.14±6.03	13.35±5.81	12.46±8.64	13.40±9.61	-0.26 (-1.2, 0.66)	0.58	0.42 (-0.49, 1.34)	0.36
vWF (%)	120.30±47.21	128.21±56.38	111.68±42.72	120.96±42.82	102.64±40.32	107.01±49.08	-0.09 (-15.3, 15.1)	0.99	-5.4 (-20.6, 9.8)	0.48
sE-selectin (ng/mL)	104.03±50.24	106.38±56.38	97.19±42.50	100.51±45.72	95.26±45.91	95.47±42.39	2.3 (-3.9, 8.5)	0.46	-1.5 (-7.6, 4.6)	0.62
sICAM-1 (ng/mL)	406.74±74.95	413.08±75.79	393.88±77.93	388.62±75.78	378.81±85.81	371.51±73.94	-10.6 (-29, 7.9)	0.26	-18.4 (-36.8, -0.1)	0.05
sVCAM-1 (ng/mL)	418.11±89.23	428.97±92.38	405.61±69.77	400.55±63.64	371.42±69.20	364.55±69.20	-17.9 (-33.7, -2.2)	0.03	-21.8 (-37.8, -5.7)	0.01

Treatment effects of pyridoxamine compared to placebo were tested. Treatment effect and mean differences were obtained using one-way ANCOVA with adjustment for baseline values. Unadjusted (crude) data are presented. For the treatment effects, adjusted mean changes (estimated marginal means) with 95% CI are given. All P values are obtained from the ANCOVA analysis with adjustment for baseline values. Skewed variables were Log transformed prior to analysis.

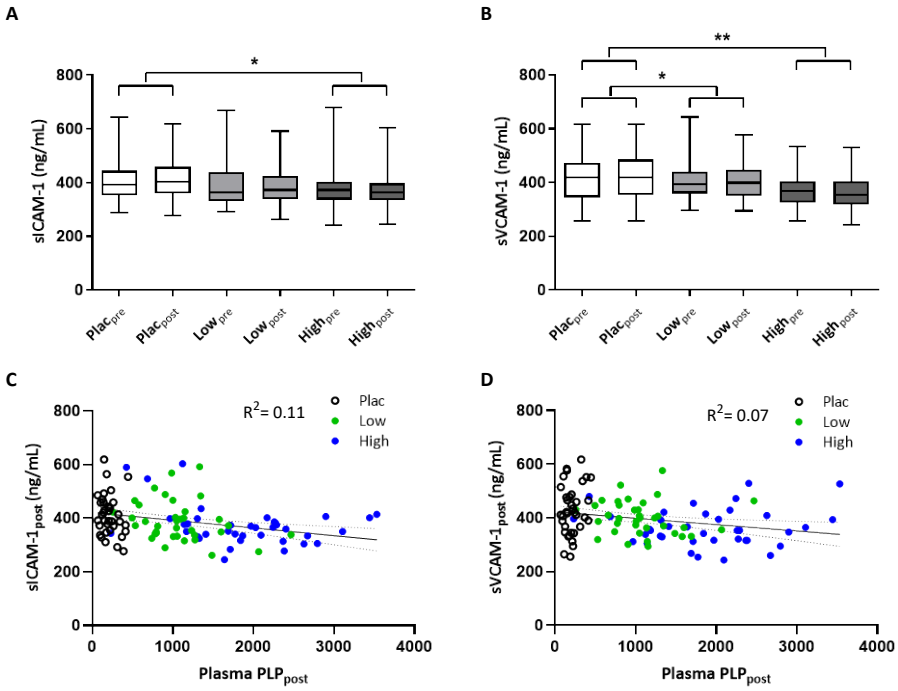

Figure 7.1 Plasma concentrations of sICAM-1 and sVCAM-1, and their association with plasma PLP concentrations after eight weeks of pyridoxamine intervention

Plasma concentrations of the endothelial function markers sICAM-1 (A) and sVCAM-1 (B) and regression analysis of the association between plasma PLP as a marker for pyridoxamine compliance and plasma levels of sICAM-1 (C) and sVCAM-1 (D). Results are given for the placebo group pre- and post-intervention (white), low dose pre- and post-intervention (grey), and high dose pre- and post-intervention (dark grey). Boxplots indicate median, 25th and 75th percentile, and min to max whiskers. The regression graph includes post-intervention results for the placebo group (white dots), low dose (green dots), and high dose (blue dots).

Differences between both pyridoxamine treatments (low and high dose) and placebo were assessed using one-way ANCOVA with adjustment for baseline values. Significance is indicated by p values obtained from the ANCOVA analysis as follows: *p≤0.05, **p<0.01. The association between PLP and sICAM-1 and sVCAM-1 levels was tested using linear regression analysis.

Discussion

In this RCT in abdominally obese individuals we found that pyridoxamine decreased plasma levels of MGO and the MGO-derived AGE MG-H1. PM was well tolerated and compliance rate was high. An eight-week treatment with PM did not improve insulin sensitivity and micro- and macrovascular function in healthy but abdominally obese individuals. We did find a significant reduction in plasma markers of endothelial dysfunction sVCAM-1 and sICAM-1.

We found a dose-dependent decrease in MGO levels over the three intervention groups. The significant reduction of plasma MGO by the high dose of pyridoxamine was around 9% from the baseline average (-22 nmol/L on average). Although the decrease of plasma MGO is modest, this effect size was also found in an RCT after supplementation with the polyphenol quercetin resulting in a decrease in plasma MGO of 10%[46] and in a well-standardized weight loss intervention study with a decrease of fasting MGO by 9%[47]. Both studies included relatively healthy abdominally obese individuals. We previously showed that MGO concentrations are associated with total, fatal, and nonfatal incident cardiovascular disease in type 1 and type 2 diabetes[12,13] with an observed difference in plasma MGO concentrations of approximately 5 to 13% between diabetic individuals with and without cardiovascular events. This means that a 9% reduction in plasma MGO as we found in this study, could be of clinical relevance. Since MGO levels in diabetes are higher than reported in the current study, PM may reduce MGO to a larger extent in individuals with overt hyperglycemia.

In line with the MGO reduction we found a reduction of the major MGO-derived AGE MG-H1. Also free plasma MG-H1, plasma protein-bound CEL, a Z-score of plasma AGEs, and the dicarbonyl compound 3-DG showed decreased levels in the high dosage group, although not significant. A lack of treatment effect on the other dicarbonyls and AGEs could be due to the small effect size or relatively large variation in fasting plasma concentrations in our study population. In addition, tissue AGEs as estimated by SAF measurements, did not change significantly after eight weeks. The lack of effect on skin AGEs is not unexpected, since SAF is associated predominantly with pentosidine and other cross-linked AGEs in skin tissue, and it generally takes months for these structural tissue changes to be altered[48,49].

MGO can lead to increased proliferation and expansion of adipose tissue, decreased adipose tissue capillarisation, and eventually insulin resistance[8,9,50-52]. Increased dicarbonyl stress, particularly in the postprandial state, can contribute to long term complications and metabolic dysregulation in obese individuals. In our previous work in high-fat diet (HFD) fed mice, we have shown that obesity-associated glucose intolerance, insulin resistance, and adipose tissue inflammation were ameliorated by a delayed intervention with PM[7]. HFD-induced increases in body weight, hyperglycemia, hypercholesterolemia, and levels of leptin and insulin were all reduced by PM. In addition, PM was associated with a general increase in glucose tolerance and insulin sensitivity. Mainly based on these data, PM may influence insulin sensitivity and β-cell function in obese individuals. Although we do see a decrease of MGO and MGO-derived AGEs,

Although we did not measure plasma MGO in our prior animal study, we found a 50% reduction of MGO in visceral adipose tissue[7]. It might be that the plasma MGO reduction of 9% by PM in this clinical study was not large enough to improve insulin sensitivity, β-cell function or vascular function measurements in our generally healthy study population. It is important, however, to note the large discrepancy between administered dosages in animal versus human studies. The dosage of PM in our animal study was at least fivefold higher compared to the high dosage in our clinical trial.

We found a significant reduction of the plasma endothelial dysfunction markers sICAM-1 and sVCAM-1, which is in agreement with literature showing that MGO is associated with endothelial dysfunction and microvascular disease in individuals with and without (pre)diabetes[53]. Previous animal work reported reduced endothelial cell viability in hyperglycemic conditions with high MGO levels[54] and AGEs have been shown to promote VCAM-1 and ICAM-1 expression in the vasculature of rabbits[55]. Animal models of diabetes also confirm an association between increased dicarbonyl stress and endothelial damage[56,57]. This was also confirmed in a study with type 1 diabetic individuals[12]. Although we did not find an effect on the other plasma markers of endothelial dysfunction, the reduction of sICAM-1 and sVCAM-1 by PM suggest a protective effect in individuals with increased levels of MGO, such as obese and diabetic individuals. The dosage and/or the duration of the intervention was most likely insufficient to improve functional measurements in the vasculature.

Safety

In addition to PM, several other scavengers of MGO and inhibitors of AGE formation have been described, including aminoguanidine[58-60] and alagebrium[61]. However, the use of these compounds were discontinued in clinical studies[5,62]. We are the first to investigate the effect of pyridoxamine in relation to metabolic and (micro)vascular outcomes in a double-blind placebo-controlled trial with abdominally obese individuals.
Preclinical experiments with PM have indicated no signs of cellular toxicity, in contrast to pyridoxine therapies[63-65]. The very low pyridoxine concentrations found after eight weeks of the high dosage indicate a limited formation of pyridoxine from pyridoxamine and hardly any plasma accumulation (Figure 7.1). This is favorable, since pyridoxine is known to be toxic in high concentrations (>25mg/day). Two other metabolites of PM, pyridoxal and pyridoxal-phosphate, show compellingly higher plasma concentrations in the treatment groups, as compared to placebo. The plasma metabolites profile was similar to our previous metabolization experiment[30].
Equally important, we did not find an increase in markers of inflammation or immune cells after the intervention, indicating an apparently safe pharmacological profile under

these circumstances. Moreover, there was no difference in reported side effects between the intervention groups and the placebo group. The reported adverse events were minimal and mostly not related to the intervention.

Strength and limitations

The design and analysis of this study has several methodological strengths, including its randomized placebo controlled doubled blind design, blinded analyses, monitoring of compliance and metabolites, very low dropout rate (<5%), and use of state of the art measurement techniques for a broad panel of metabolic markers, insulin metabolism, and (micro)vascular measurements. We showed good compliance based on self-reported feedback during the intervention, amount of returned capsules, and plasma and urine concentrations of pyridoxamine and its metabolites. Our study was successfully randomized, with comparable groups at baseline.

In the analysis of this clinical trial we also faced several limitations. For safety reasons, we designed this trial with a moderate dosage of pyridoxamine. Furthermore, our population could be labeled as apparently healthy, but at risk of developing metabolic complications. As a consequence, the pyridoxamine concentration in our study was possibly too low to detect other metabolic changes and our population was possibly too healthy to allow for significant improvements in vascular function. An intervention in diabetic individuals might show larger effects on these outcome parameters. Furthermore, a lack of statistical significance in the other secondary outcome measurements could arguably be due to a lack of power.

We performed measurements before the intervention at baseline, and after the intervention at follow-up. Therefore we lack temporal information of the treatment effects. It would be interesting to study acute changes or possible time related effects after onset of the pyridoxamine treatment. However, to evaluate this, a potential follow-up study with a longer duration would be more suitable.

Conclusion

Pyridoxamine was shown to be safe and metabolically active in abdominally obese individuals. Pyridoxamine did not affect insulin sensitivity or vascular function in this study population. However, the reduction in MGO, glycation markers, and endothelial dysfunction markers in relatively healthy individuals provides a good basis for future research. Whether pyridoxamine will be potent enough to improve metabolic markers and vascular function in (pre) diabetic individuals or people suffering from CVD remains to be established.

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Supplemental materials

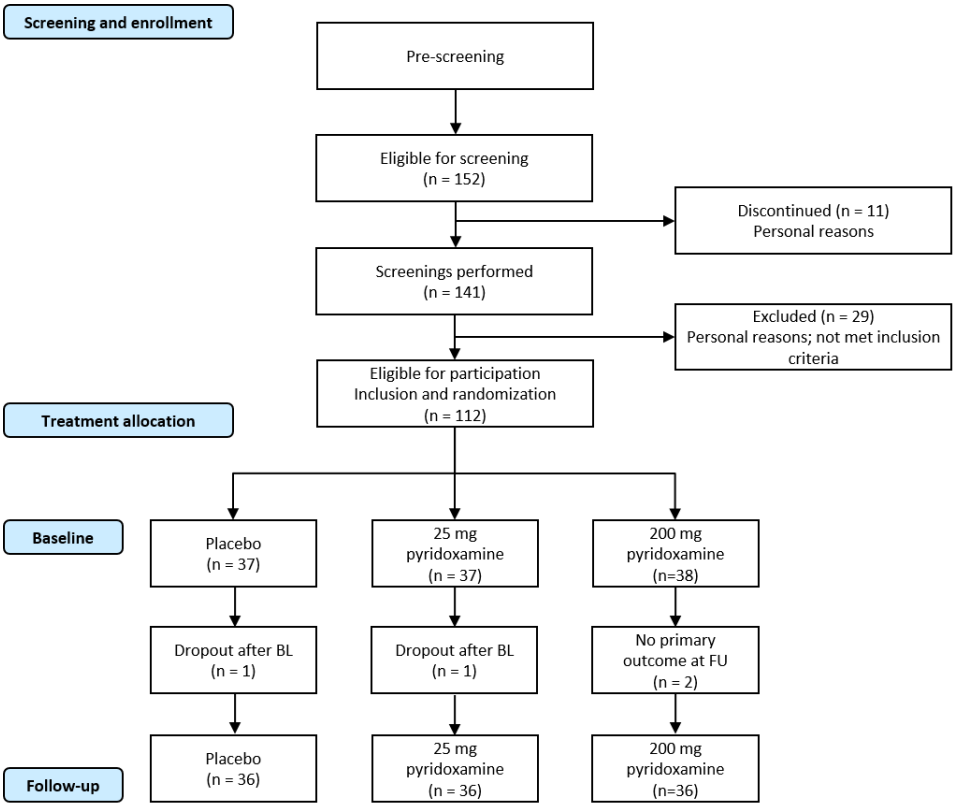

Figure S7.1 Flow chart of study enrolment.
After pre-screening, 152 applicants were eligible for screening and in the end 141 individuals signed informed consent and were screened. Of these, 29 individuals did not meet all inclusion criteria or reconsidered and dropped out because of personal reasons before onset of the intervention. Finally, 112 individuals met the inclusion criteria and were randomized for participation in the trial. All 112 individuals completed baseline measurements, 37 individuals were randomly assigned to the placebo group, 37 to the low dose group, and 38 to the high dose group. One person dropped out of the placebo group due to a medical emergency, one person dropped out of the low dose group because of personal reasons, and 2 individuals in the high dose group did not complete the primary outcome measurement due to unforeseen circumstances during the measurement day. At the end of the study, 36 individuals per group had finished all primary outcome measurements at the follow-up visit.

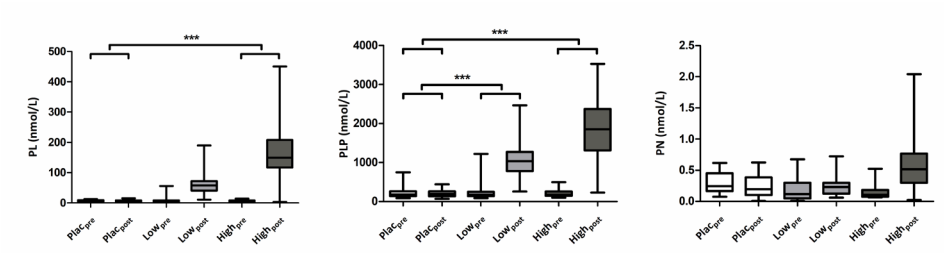

Figure S7.2 Pyridoxamine metabolites in plasma before and after pyridoxamine treatment.
Plasma concentrations of the pyridoxamine metabolites pyridoxal (PL), pyridoxal-phosphate (PLP) and pyridoxine (PN) are given for the placebo group pre- and post-intervention (white), low dose pre- and post-intervention (grey), and high dose pre- and post-intervention (dark grey). Boxplots indicate median, 25th and 75th percentile, and min to max whiskers. Differences between both pyridoxamine treatments (low and high dose) and placebo were assessed using one-way ANCOVA with adjustment for baseline values. Significance is indicated by p values obtained from the ANCOVA analysis: ***p<0.001. Skewed variables were Log transformed prior to analysis.

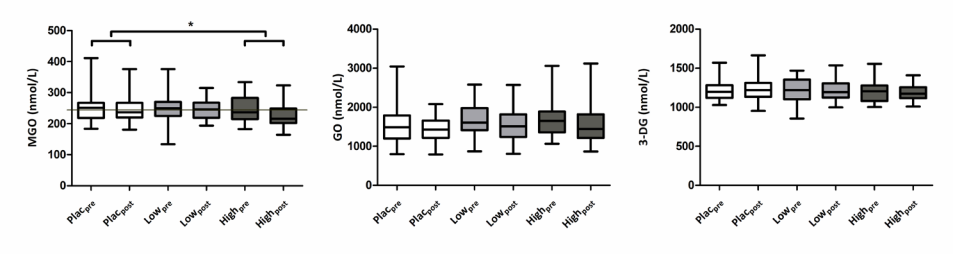

Figure S7.3 Plasma dicarbonyls before and after pyridoxamine treatment.
Plasma concentrations of dicarbonyl compounds methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG) are given for the placebo group pre- and post-intervention (white), low dose pre- and post-intervention (grey), and high dose pre- and post-intervention (dark grey). Boxplots indicate median, 25th and 75th percentile, and min to max whiskers. Differences between both pyridoxamine treatments (low and high dose) and placebo were assessed using one-way ANCOVA with adjustment for baseline values. Significance is indicated by p values obtained from the ANCOVA analysis: *p<0.05.

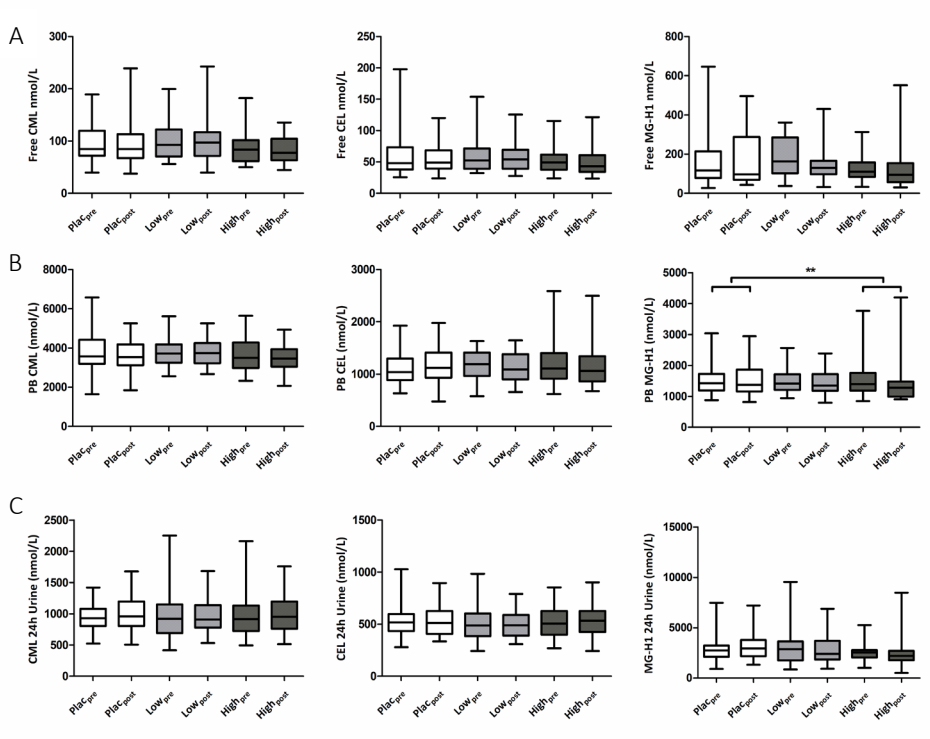

Figure S7.4 Plasma and urinary AGEs.

Concentrations in nmol/L of the free plasma AGE CML, CEL, and MG-H1 (panel **A**), protein-bound AGEs (PB) CML, CEL and MG-H1 in plasma (panel **B**), and free CML, CEL and MG-H1 in 24h urine samples (panel **C**). From left to right, results are given for the placebo group pre- and post-intervention (white), low dose pre- and post-intervention (grey), and high dose pre- and post-intervention (dark grey). Boxplots indicate median, 25th and 75th percentile, and min to max whiskers. Differences between both pyridoxamine treatments (low and high dose) and placebo were assessed using one-way ANCOVA with adjustment for baseline values. Significance is indicated by p values obtained from the ANCOVA analysis: **p<0.01.

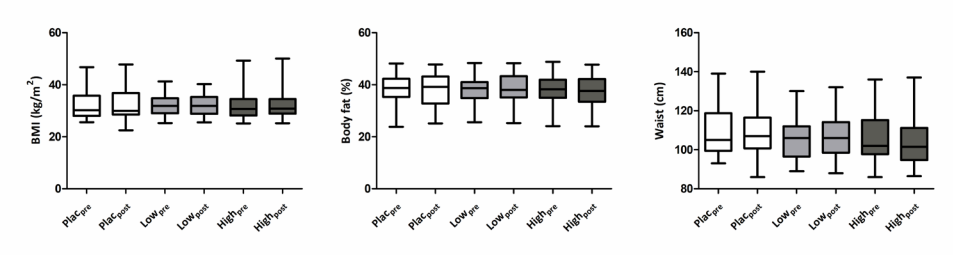

Figure S7.5 Anthropometric markers.
BMI, body fat percentage, and waist circumference were unchanged after intervention. From left to right, results are given for the placebo group pre- and post-intervention (white), low dose pre- and post-intervention (grey), and high dose pre- and post-intervention (dark grey). Boxplots indicate median, 25th and 75th percentile, and min to max whiskers. Differences between both pyridoxamine treatments (low and high dose) and placebo were assessed using one-way ANCOVA with adjustment for baseline values.

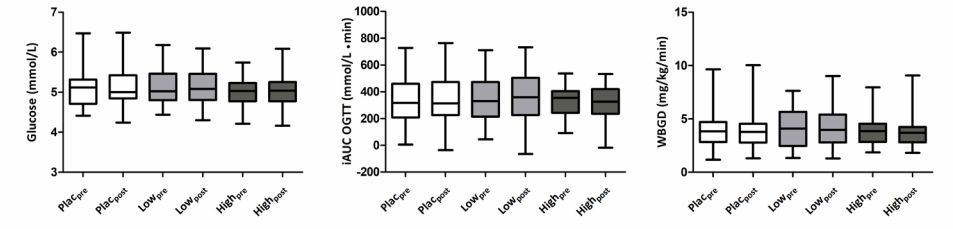

Figure S7.6 Glucose and clamp data.
Fasting glucose, glucose iAUC (incremental area under the curve), and WBGD (whole body glucose disposal) as measured with the euglycemic hyperinsulinemic clamp, were unchanged after intervention. From left to right, results are given for the placebo group pre- and post-intervention (white), low dose pre- and post-intervention (grey), and high dose pre- and post-intervention (dark grey). Boxplots indicate median, 25th and 75th percentile, and min to max whiskers. Differences between both pyridoxamine treatments (low and high dose) and placebo were assessed using one-way ANCOVA with adjustment for baseline values.

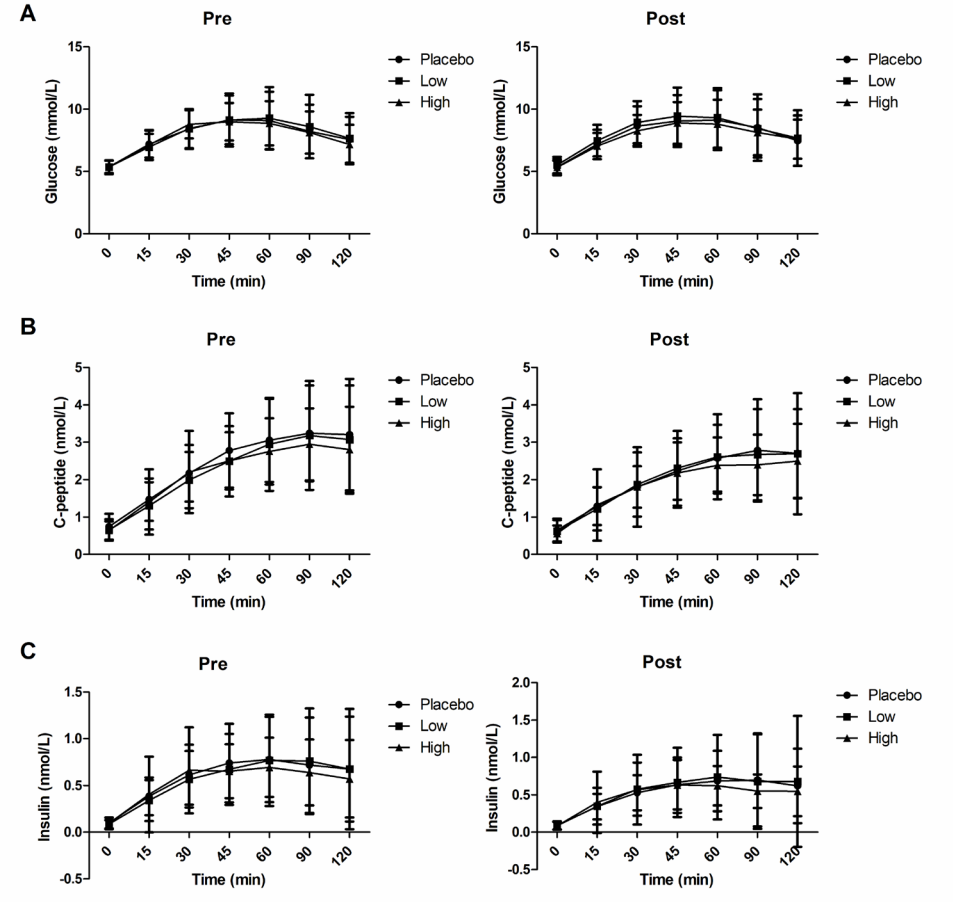

Figure S7.7 Glucose, insulin, and C-peptide time curves as measured during the OGTT.
Glucose (A), C-peptide (B) and insulin (C) concentrations as assessed with a two-hour seven-sample oral glucose tolerance test (OGTT). Graphs per panel (A, B and C) display pre-intervention levels (left) and post-intervention levels (right). Differences between the study groups in repeated measures during the OGTT were analyzed using mixed ANOVA with intervention as between-subjects factor and time within-subjects factor. For the mixed ANOVA intervention analysis, the baseline postprandial curve was subtracted from the postprandial curve at follow-up.

Table S7.1 Pyridoxamine metabolites after 8 weeks of intervention in comparison to placebo.

	Crude data						Treatment effect			
	Placebo BL Mean±SD	Placebo FU Mean±SD	Low BL Mean±SD	Low FU Mean±SD	High BL Mean±SD	High FU Mean±SD	PM low dose - placebo Mean change (95% CI)	p *value*	PM high dose - placebo Mean change (95% CI)	p *value*
Pyridoxal (PL)	6.39±3.83	6.07±3.22	8.54±10.78	61.34±34.54	6.48±3.21	271.70±373.35	0.99 (0.82, 1.2)	<0.0001	1.5 (1.3, 1.7)	<0.0001
Pyridoxal-phosphate (PLP)	229.93±146.50	211.32±103.45	245.84±235.52	1071.95±443.06	209.25±90.65	1879.39±773.18	0.72 (0.62, 0.81)	<0.0001	0.95 (0.86, 1)	<0.0001
Pyridoxin (PN)	0.24±0.18	0.24±0.18	0.22±0.29	0.26±0.20	0.15±0.14	3.35±12.59	-0.04 (-0.4, 0.3)	0.81	0.44 (0.12, 0.76)	0.01

Treatment effects of pyridoxamine compared to placebo were tested. Treatment effect and mean differences were obtained using one-way ANCOVA with adjustment for baseline values. Unadjusted (crude) data are presented. For the treatment effects, adjusted mean changes (estimated marginal means) with 95% CI are given. All P values are obtained from the ANCOVA analysis with adjustment for baseline values. Skewed variables PL, PLP, and PN, were Log transformed prior to ANCOVA analysis.

Table S7.2 Treatment effects of 8 weeks pyridoxamine on fatty liver index, eGFR and blood pressure

	Crude data						Treatment effect			
	Placebo BL Mean±SD	Placebo FU Mean±SD	Low BL Mean±SD	Low FU Mean±SD	High BL Mean±SD	High FU Mean±SD	PM low dose - placebo Mean change (95% CI)	*P value*	PM high dose - placebo Mean change (95% CI)	*P value*
FLI (fatty liver index)	12.08±17.28	11.97±18.02	7.78±9.40	8.42±9.71	10.47±17.06	11.16±19.13	0.66 (-1.3, 2.7)	0.52	0.77 (-1.2, 2.7)	0.43
eGFR (mL/min/1.73m^2)	93.02±19.30	93.79±18.14	89.12±14.09	88.89±14.06	98.00±15.30	97.62±15.81	-0.88 (-4.3, 2.6)	0.62	-0.001 (-3.4, 3.4)	1.0
24h SBP (mmHg)	122.53±10.87	124.11±12.17	123.08±10.99	125.83±10.04	124.37±11.75	126.03±12.97	1.5 (-1.8, 4.8)	0.37	0.42 (-2.9, 3.7)	0.8
24h DBP (mmHg)	77.83±7.62	78.46±8.27	78.35±7.70	79.69±7.22	77.97±11.14	79.03±11.31	1.0 (-1.03, 3.06)	0.33	0.72 (-1.3, 2.8)	0.49
24h MAP (mmHg)	98.22±8.28	99.43±9.19	98.73±8.64	100.75±7.98	99.34±11.00	100.53±11.42	1.15 (-1.3, 3.6)	0.36	0.34 (-2.1, 2.8)	0.79

Treatment effects of pyridoxamine compared to placebo were tested. Treatment effect and mean differences were obtained using one-way ANCOVA with adjustment for baseline values. Unadjusted (crude) data are presented. For the treatment effects, adjusted mean changes (estimated marginal means) with 95% CI are given. All P values are obtained from the ANCOVA analysis with adjustment for baseline values.

Table S7.3 Treatment effects on immune cell counts in circulating blood.

	Crude data						Treatment effect			
(%) Total cells	**Placebo BL Mean±SD**	**Placebo FU Mean±SD**	**Low BL Mean±SD**	**Low FU Mean±SD**	**High BL Mean±SD**	**High FU Mean±SD**	**PM low dose - placebo Mean change (95% CI)**	**p *value***	**PM high dose - placebo Mean change (95% CI)**	**p *value***
Lymfocytes	30.87±7.30	30.00±7.97	29.97±7.89	30.28±8.15	32.61±5.58	32.95±6.13	0.076 (-0.077, 0.23)	0.33	0.13 (-0.021, 0.28)	0.09
Granulocytes	58.19±7.25	59.53±8.72	58.03±8.24	57.44±7.97	54.74±10.16	55.53±6.42	-0.13 (-0.54, 0.27)	0.52	-0.079 (-0.48, 0.32)	0.70
Monocytes	8.14±1.87	7.67±2.03	8.64±1.78	8.58±2.43	8.29±1.97	8.16±1.59	0.046 (-0.006, 0.097)	0.08	0.028 (-0.022, 0.079)	0.27
Eosinophils	2.43±0.87	2.39±1.13	2.94±1.69	3.28±2.44	2.79±1.82	2.84±1.65	0.039 (-0.02, 0.097)	0.19	0.003 (-0.054, 0.059)	0.93
Basophils	0.78±0.42	0.61±0.49	0.89±0.32	1.06±0.23	0.87±0.41	0.71±0.46	0.026 (0.015, 0.038)	<0.001*	0.003 (-0.008, 0.014)	0.63

Treatment effects of pyridoxamine compared to placebo were tested. Treatment effect and mean differences were obtained using one-way ANCOVA with adjustment for baseline values. Unadjusted (crude) data are presented. For the treatment effects, adjusted mean changes (estimated marginal means) with 95% CI are given. All P values are obtained from the ANCOVA analysis with adjustment for baseline values. Skewed variables were Log transformed prior to analysis. *The effect on the basophil count (*) in the low dose group, which is not apparent in the high dose group, is most likely a chance finding unrelated to the PM intervention.*

Table S7.4 Habitual dietary intake of AGEs and dicarbonyls before onset of the intervention.

	Placebo	Low dose	High dose
Dietary AGEs			
dCML (mg/day)	3.63 ± 1.46	3.95 ± 1.65	4.46 ± 1.92
dCEL (mg/day)	3.37 ± 1.27	3.62 ± 1.60	3.94 ± 1.78
dMG-H1 (mg/day)	24.60 ± 10.67	27.64 ± 11.31	28.17 ± 11.37
Dietary dicarbonyls			
dMGO (mg/day)	3.42 ± 1.37	3.88 ± 1.53	3.53 ± 1.22
dGO (mg/day)	3.23 ± 1.42	3.68 ± 1.49	3.72 ± 1.50
d3-DG (mg/day)	17.42 ± 14.26	19.01 ± 21.11	21.48 ± 15.08

Habitual dietary intake of AGEs and dicarbonyls in mg/day, assessed by FFQ (food frequency questionnaire). Data are means ± SD at baseline.

Chapter 8

Pyridoxamine does not reduce arterial stiffness in an eight-week randomized placebo-controlled intervention trial with abdominally obese individuals

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In preparation for publication

Chapter 9

Summary and general discussion

Impact statement

Summary and general discussion

Advanced glycation endproducts (AGEs) and dicarbonyl compounds play an important role in the development and progression of many chronic metabolic diseases. Intervention strategies targeting the glycation pathway have been described previously (Table 1.1 general introduction). In this thesis we investigated different intervention approaches in clinical trials with regard to their potential to reduce protein glycation and dicarbonyl stress.

Lifestyle interventions may reduce dicarbonyl stress in the body. We have investigated a weight loss intervention by caloric restriction and the effect of physical activity (including a high intensity exercise intervention) in an older population. Some pharma- and nutraceutical interventions are able to decrease dicarbonyl and AGE levels, by direct scavenging or by the induction of detoxification pathways like GLO1. In this thesis, we evaluated the effect of two flavonoids (quercetin and epicatechin) and the B6 vitamer pyridoxamine. Other strategies and pharmaceuticals exist that can reduce dicarbonyl or AGE levels (Table 1.1 general introduction), such as GLO1 inducers[1,2], and dietary AGE interventions[3], but these were not examined in this thesis.

Dicarbonyls and AGE in obesity

Dicarbonyl compounds like methylglyoxal and glyoxal can react with proteins, lipids, and DNA, leading to the formation of AGEs, ultimately resulting in cellular dysfunction and tissue damage. Obesity is associated with higher dicarbonyl stress[4], which can contribute to the development of various obesity-related problems such as insulin resistance, microvascular pathologies like diabetic retinopathy and nephropathy, and cardiovascular disease[5]. A severe dysregulation of white adipose tissue, increased production of reactive oxygen species (ROS) and a decrease in GLO1 activity can all contribute to increased dicarbonyl stress[4]. Increased MGO levels may also directly affect adipocity and insulin sensitivity[6,7].

In **chapter 2** we found that postprandial plasma MGO, GO and 3-DG levels were significantly higher in abdominally obese men, compared to lean men. This finding confirms previous data from (morbidly) obese women with diabetes[8]. We found that large amounts of dicarbonyl compounds are repeatedly produced during postprandial glucose spikes and are increased in obese men[9]. This increase in dicarbonyl compounds may contribute to complications associated with obesity; this is the reason why we search for novel and early interventions in this population.

Except for CML, plasma AGE levels and skin autofluorecence (SAF) were not different between lean and obese individuals, which could be an indication of a more limited downstream effect in the glycation pathway at this stage of the pathology (i.e. an obese

population at risk, but without diabetes or other cardiometabolic diseases). However, this does not exclude that there is no AGE accumulation in tissue of obese individuals. The decrease in plasma CML in obese men (chapter 2) is in line with the uptake of CML in adipose tissue in obesity as previously described[10].

Weight loss

Our study in chapter 2 showed that weight loss in an abdominally obese population reduced dicarbonyl stress, especially postprandial levels. Plasma dicarbonyl concentrations approached or even subceeded the levels from lean individuals. Likewise, protein-bound CEL was reduced, but other AGEs and SAF were not affected by the intervention.

Glucose is a major substrate for the formation of dicarbonyls and AGEs[9], but lipid peroxidation and reactive oxygen species may also contribute[11]. Indeed, we confirmed that the effect on glucose levels explains a large part of the treatment effect on dicarbonyl stress. A reduction in glycation and lipoxidation reactions, as a consequence of caloric restriction, has also been suggested as a possible explanation for a reduction in glycation markers in a Japanese study on caloric restriction[12]. It is also possible that caloric restriction could activate the glyoxalase system, possibly via activation of Nrf2[13], although we did not find indications for this underlying mechanism. An increase in ketone bodies such as acetoacetate and beta-hydroxybutyrate, which can scavenge MGO[14], are other possible reasons for the decrease in dicarbonyls.

Physical activity

The results of physical activity and exercise as intervention strategies in the glycation pathway are more complicated to interpret. A reduction of sedentary time and increase in general activity has shown to be strongly related with glycemic control and insulin sensitivity[15,16], while increased physical exercise is mostly associated with vascular health[17]. However, human studies assessing the effect of physical exercise on dicarbonyl stress or AGEs have yielded conflicting results[18-22]. Specific intensity and duration of the exercise interventions, in addition to population differences, are a possible explanation for the observed discrepancies.

In our study, as presented in chapter 3, we showed that active individuals had higher levels of plasma CML than sedentary individuals. Moreover, we found a positive association between CML and VO_2 max. It is possible that exercise promotes tissue repair and the breakdown of vessel wall cross-links, leading to higher levels of circulating protein-bound AGEs[23,24]. Additionally, higher metabolic rate and higher energy intake,

resulting in higher levels of formation and intake of dietary AGEs[25], may also explain the increased levels of CML.

Our findings were not in line with our prior hypothesis that exercise could decrease dicarbonyl stress, and are in conflict with earlier research by our group comparing dicarbonyl stress and AGE levels in lifelong endurance athletes and sedentary controls[26]. Athletes had lower levels of dicarbonyls and MG-H1, while CML and CEL were increased[26]. The overall decrease in dicarbonyl stress appeared to be a favorable consequence of physical exercise. In our study in chapter 3, we can only confirm the increased CML concentrations related to physical activity. Furthermore, we showed that a high intensity interval training (HIIT) intervention did not materially change any plasma dicarbonyl or AGE concentrations, indicating the need for other approaches to intervene in the glycation pathway.

Flavonoid intervention

In chapter 4 we demonstrated that the flavonoid quercetin led to a reduction in plasma levels of MGO in (pre)hypertensive adults. No significant effects were observed for AGEs, glyoxal, or 3-deoxyglucosone. The administration of epicatechin did not result in a significant decrease in either dicarbonyl levels or AGEs.

This report was in line with earlier experimental work showing that quercetin could scavenge MGO[27,28]. In contrast to MGO, we could not reproduce the previous findings that quercetin or epicatechin also inhibit AGE formation[29-32]. Quercetin probably exerts its lowering effect on MGO levels via biochemical scavenging, as we did not find any effects on glucose levels or glyoxalase 1 activity. It should be noted that rapid modifications occur during first-pass metabolism of flavonoids and conjugated forms of these flavonoids may have an impact on bioactivity[33,34]. Many experimental studies have utilized unconjugated flavonoid forms, or supra-physiological dosages, which may explain discrepancies between experimental models and in vivo animal studies. Furthermore, in the same study, we found that supplementation of quercetin significantly decreased sE-selectin, IL-1β, and a z-score of inflammation markers[35]. Unfortunately, we lacked statistical power to study a possible effect of MGO reduction by quercetin on the inflammation markers.

Pyridoxamine intervention

The B6 vitamer pyridoxamine has been shown to scavenge MGO and inhibit AGE formation in several experimental studies[36-39]. Preclinical and clinical studies also showed promise, as large-scale clinical trials with PM showed modest improvements of eGFR in patients with nephropathy[40,41]. Furthermore, in a previous mouse model of obesity, we

showed that pyridoxamine improves glucose and insulin metabolism, and prevents adipose tissue inflammation and vascular dysfunction[42]. However, no human intervention studies had investigated a possible role of pyridoxamine in the prevention of metabolic and vascular dysfunction. In our clinical trial, as described in chapters 5 to 8, we have studied the effect of pyridoxamine on MGO in abdominally obese individuals, as well as the effects on glycation, insulin sensitivity, and micro- and macrovascular function.

In chapter 6 we optimized and validated a method for the quantification of vitamin B6 in plasma and urine using UPLC-MS/MS. After a metabolization experiment, we demonstrated that pyridoxamine supplementation offers an advantage over current B6 supplements. We showed that pyridoxamine supplementation resulted in high levels of pyridoxal 5'-phosphate, the biologically active form of vitamin B6. Formation of pyridoxine was minimal. Pyridoxine is currently the most used B6 supplement and is the main cause of vitamin B6 toxicity. High levels of pyridoxin are neurotoxic, and this form of B6 is also a competitive inhibitor of pyridoxal 5'-phosphate[43-46]. Combined with the data on glycation, this could make pyridoxamine a more attractive supplement regarding both the treatment of metabolic diseases and vitamin B6 deficiency. Due to the rapid metabolization, it should be noted that the three daily dose resulted in a more constant plasma concentration during the day.

In chapter 7, we showed that pyridoxamine treatment effectively reduced plasma levels of MGO and the MGO-derived AGE MG-H1. The decrease in MGO was dose-dependent, with the high dosage of pyridoxamine leading to a 9% reduction in plasma MGO compared to baseline levels. While this reduction appears modest, it is comparable to previous studies as discussed below. Furthermore, we observed a non-significant reduction in other AGEs, as indicated by the borderline significant z-score of AGEs. Pyridoxamine did not improve insulin sensitivity or vascular function in abdominally obese participants. Based on our previous experimental animal work, we expected to find some positive effect on glucose metabolism, whole-body insulin sensitivity or microvascular function. A possible explanation for these null findings is that HFD fed mice are a more extreme model, and the dosage of pyridoxamine in our animal study was fivefold higher as compared to the highest dosage in our clinical trial. The treatment effect in our overweight population was probably too low to affect a functional outcome measurements. Notably, there was a significant reduction in plasma markers of endothelial dysfunction, specifically soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble intercellular adhesion molecule-1 (sICAM-1). In accordance, previous studies show that MGO is associated with endothelial dysfunction and damage, as indicated by increased levels of VCAM-1 and ICAM-1 expression[47-49]. Therefore, we conclude that there is an improvement of endothelial dysfunction by pyridoxamine, although we did

not yet observe changes in microvascular readouts, as was shown in individuals with and without (pre)diabetes[50].

In **chapter 8**, we showed that arterial stiffness was not influenced by pyridoxamine treatment over the eight-week time period. We thoroughly investigated arterial stiffness by carotid distensibility, pulse wave velocity, and carotid intima media thickness. It has been described that AGEs are associated with several markers of arterial stiffness[51-53] and a decrease in MGO might lead to less MGO-derived cross-linking of collagen in the arterial wall and less arterial stiffness[51-53]. It might be that the effect of pyridoxamine on MGO was most likely too small to detect effects on arterial stiffness.

Common ground between the interventions

Reduction in plasma methylglyoxal

Weight loss, quercetin, and pyridoxamine interventions all showed an effect of approximately 9-10% reduction in plasma MGO in the intervention group as compared to the placebo group. It seems that this treatment effect of 10% on plasma MGO is a recurring observation for our relatively healthy population. Furthermore, the flavonoid hesperidin also showed an approximately 10% reduction in plasma MGO (unpublished). We can not exclude the possibility that the reduction of MGO in tissues is larger than 10% . Furthermore, it would be of interest to investigate if we can reduce plasma dicarbonyls more in individuals with diabetes or in patients with higher baseline levels of plasma dicarbonyls, such as observed in renal failure.

Effect on AGEs, pentosidine and skin autofluorescence

Both in the pyridoxamine and weight loss intervention we found an effect on MGO and the MGO derived AGEs MG-H1 and CEL. In the flavonoid intervention study, quercetin only affected MGO levels.

In the flavonoïd and weight loss study, pentosidine was unchanged. In the weight loss and pyridoxamine intervention SAF was also unchanged. Skin autofluorescence measurements are associated with pentosidine[54], as was previously described in the Maastricht study[53], and also found in the weight loss study (data not shown). The lack of effect on long-lived AGEs, like the cross-link pentosidine, or skin autofluorescence (SAF), was in line with our expectations because the formation of AGE cross-links like pentosidine are generally formed very slowly. Indeed, AGE and cross-link formation in tissue, are not immediately affected by (short-term) changes in plasma dicarbonyls or weight loss as previously described[55,56].

Methodological considerations

Our population, with the exception of the exercise study, all included relatively healthy individuals at risk of developing metabolic or vascular complications. In the weight loss trial and pyridoxamine study we included abdominally obese individuals on the basis of abdominal fat. The flavonoid study included (pre)hypertensive individuals and the exercise intervention was performed in older active and sedentary individuals. Although most of our studies have been carried out in individuals at risk of developing obesity- or vascular-related complications, we cannot definitively extrapolate our findings to a diabetic (or any other) population. However, forthcoming studies in a diabetic or more severely obese population, with higher baseline levels of dicarbonyls and AGEs, could provide more room for treatment effects.

It would be interesting to see if a reduction of glycation markers (for example by pyridoxamine) could influence disease outcome in a prospective investigation. The factors time, dosage and disease status are vital parameters for future investigations. As we have found no negative side-effects of pyridoxamine or flavonoids, an increased dosage or longer duration is reasonable. The duration of the nutraceutical interventions was only four weeks for the flavonoid intervention and eight weeks for the pyridoxamine intervention. These are relatively short intervention periods.

In terms of microvascular dysfunction, it is important to discriminate between the different measurement techniques that were used to assess this broad spectrum of function. We performed endothelial function measurements in the skin (skin laser doppler flowmotion analysis), brachial artery (flow mediated dilation), muscle (insulin mediated microvascular recruitment), and in plasma samples. However, this does not exclude changes in other vascular beds, sensitive to dicarbonyl stress, like the eye, brain and kidney. It is possible that these tissues have more potential for improvement, since these are generally the first to suffer from metabolic and consequent vascular disorders in obesity.

It should be noted that we describe dicarbonyl and AGE formation primarily in the context of hyperglycemia, as sugars are a major source for their formation. However, also inflammation, hypoxia and increased oxidative stress, are major contributors to dicarbonyl formation and the glycation pathway. In obesity, the combination of hyperglycemia, hyperlipidemia, low energy expenditure, low grade-inflammation and hypoxic hypertrophic adipose tissue, can all increase dicarbonyl and AGE formation.

A common complication of obesity is non-alcoholic fatty liver disease (NAFLD). Increased levels of free fatty acids are an important factor in obesity and in the development of microvascular complications. Although we did evaluate free fatty acids and the fatty liver index, due to practical considerations and the focus on glycation, NAFLD is not further examined in the context of this thesis.

Intervention studies have several limitations, many of which can be tackled in well-designed, blinded, randomized placebo-controlled trials. Maintaining homogeneity in the study population is a common difficulty for intervention studies. Very strict inclusion criteria on the other hand, limit the translational potential of the resulting intervention effect. The weight loss intervention in chapter 2 was performed in abdominally obese men for example, while the effect of physical activity in chapter 3 was examined in an older population of men and women. The flavonoid intervention in chapter 4 and pyridoxamine intervention in chapters 6-8 also included men and women, both studies with different inclusion criteria as described per chapter. Stratified randomization (for age and gender in the pyridoxamine trial for example) is sometimes warranted.

Due to the high cost and large burden for the participants, intervention studies are often restricted by a relatively short study duration and limited statistical power. Another difficulty for intervention studies is monitoring the compliance, the adherence to the interventional product. Compliance can be measured in several ways as described in chapter 5 and 7 for the pyridoxamine trial. However, direct observations are often lacking and intention-to-treat analysis, disregarding compliance data, is therefore advised.

Several strengths of the performed trials in this thesis include the use of randomized placebo-controlled trial designs, blinded analyses, intensive monitoring of the participants, low-dropout rates, and the use of validated UPLC-MS/MS measurements for our large panel of AGEs and dicarbonyl compounds. Furthermore, we included a very large panel of metabolic and vascular function measurements in the pyridoxamine trial.

Clinical relevance

We know from experimental studies that MGO is a potential mediator in obesity[57] and that MGO can accumulate in adipose tissue[42]. Elevated MGO levels in adipose tissue are associated with the decreased capillarization in adipose tissue and with the development of insulin resistance[58,59]. The rapid formation of MGO in the postprandial state could play a role in the development of metabolic and vascular complications in obesity.

Previous data from our group showed that MGO is associated with total, fatal, and non-fatal incident cardiovascular disease in type 1 and type 2 diabetes[60,61]. In these studies, differences in plasma MGO concentrations of approximately 5 to 13 % were reported between diabetic individuals with and without cardiovascular events. This indicates the possible clinical relevance of a 10% reduction in methylglyoxal, as we have found in our intervention trials with pyridoxamine, quercetin, and weight loss.

Dicarbonyl stress, insulin sensitivity and vascular function appears to be (at least in part) reversible by weight loss[62-64]. A causal relation between the reduction in dicarbonyl stress and glycation markers, and insulin sensitivity and vascular function markers,

remains to be established, as we did not find these effects after eight weeks of pyridoxamine.

Overview of the intervention effects on dicarbonyls and AGEs as studied in this thesis				
	Weight loss Chapter 2	Physical exercise Chapter 3	Flavonoid study Chapter 4	Pyridoxamine study Chapter 6-8
Dicarbonyls	Fasting MGO: 10%↓ Postprandial MGO, GO & 3-DG ↓	MGO, GO, 3-DG: no effect	Fasting MGO: 10%↓ GO, 3-DG: no effect	Fasting MGO: 9%↓ GO, 3-DG: no effect
AGEs	CEL ↓ CML, MG-H1: no effect	CML ↑ CEL, MG-H1: no effect	CML, CEL, MG-H1: no effect	MG-H1 ↓ Z-score of AGEs ↓

Intervention effects of the weight loss, physical activity, flavonoid, and pyridoxamine study on dicarbonyls and AGEs. Increased (↑) and decreased (↓) plasma concentrations of dicarbonyls methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG), and of the AGEs Nε-(carboxyethyl)lysine (CEL), Nε-(carboxymethyl)lysine (CML) and Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) are shown. The intervention effects are corrected for the placebo (or control) group. For the fasting MGO concentrations, the placebo-corrected decrease from the start of the intervention is shown in percentages.

Impact statement

Future perspectives and scientific impact

The treatment of multifactorial chronic diseases like diabetes is a continuing struggle. In recent years large advances have been made. In diabetes, treatments with metformin, GLP-1 receptor agonists, and SGLT-2 inhibitors, have greatly progressed disease management[65,66]. However, the increasing prevalence of diabetes, accompanied by many micro- and macrovascular complications, requires a continuous improvement of treatments and more focus on prevention and personalized healthcare, in combination with improved self-management[67].

As mentioned in the introduction, the WHO states, "*Diabetes can be treated and its consequences avoided or delayed with diet, physical activity, medication and regular screening and treatment for complications*". In our research we search for new perspectives and new interventions strategies in the glycation pathway.

A reduction in glycation could relieve part of the burden of obesity and diabetes. However, there are currently no clinical implementations of treatments focusing on the excessive endogenous production of dicarbonyls and consequent accumulation of advanced glycation endproducts (AGEs). In our research we studied the effect of a weight loss, exercise, flavonoid, and pyridoxamine intervention trial for their potential as strategies to inhibit the glycation pathway.

The treatment of complex metabolic diseases without a single molecular target and many interrelated pathways is a difficult feat. When focusing on the reduction of dicarbonyl species and AGEs, we found many reassuring effects in our intervention studies. These clinical studies, as described in this thesis, show that it is possible to reduce dicarbonyl stress and glycation in humans, without apparent side effects. Pyridoxamine and quercetin both show a 10% reduction in plasma MGO, accompanied by positive effects on markers of inflammation and endothelial dysfunction. Pyridoxamine also shows promise with regard to the inhibition of AGEs, but we did not find effects on insulin sensitivity or vascular function. In comparison, the effect size of MGO reduction by quercetin and pyridoxamine of 10% is similar to that what we found in our weight loss intervention. Our weight loss intervention study shows significant effects on fasting MGO and postprandial MGO levels. In contrast, high intensity exercise did not reduce glycation markers.

Many cohort and experimental studies have established the relation between glycation markers and vascular complications. It is reassuring that we were able to reduce dicarbonyl stress and glycation markers in our clinical studies. These data serve as a basis for future interventions; the focus could thereby shift towards treatment effects on

metabolic and vascular function as a consequence of lower dicarbonyl stress. Furthermore, higher dosages and longer duration of treatment should be considered in order to improve efficacy. Individuals with increased MGO stress, such as in diabetes, could benefit the most.

With regard to the prevention and treatment of diabetic complications, I believe the most important question is that of effect size. The evidence showing associations between dicarbonyl stress, AGEs, and disease progression is abundant and irrefutable[57,60,68]. Based on the studies described in the thesis, we now know it is possible to effectively target MGO in apparently healthy individuals. The question remains what the treatment effect of a nutraceutical or lifestyle intervention on plasma MGO should be, to achieve an improved disease outcome in the long term.

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Addendum

Nederlandse samenvatting

Dankwoord

CV and Scientific output

Nederlandse samenvatting

Advanced glycation endproducts (AGE's) en dicarbonylverbindingen spelen een belangrijke rol in de ontwikkeling en voortgang van chronische metabole ziekten. In dit proefschrift hebben we verschillende interventiestrategiën onderzocht in klinische (humane) studies om te kijken of we deze AGE's en dicarbonyls kunnen verlagen. Met name gewichtsverlies door calorische restrictie, lichamelijke activiteit en farmaceutische interventies met flavonoïden en pyridoxamine zijn beschreven in dit proefschrift.

AGE's en dicarbonyls in obesitas

Obesitas is geassocieerd met verhoogde concentraties van dicarbonyls, voornamelijk veroorzaakt door verhoogde suikerwaarden. Dicarbonyls kunnen reageren met aminozuren uit eiwitten, lipiden en DNA, wat leidt tot de vorming van AGE's en bijgevolg verstoorde celfuncties en weefselschade. Deze schadelijke modificaties dragen bij aan de ontwikkeling van verschillende obesitas en diabetes gerelateerde problemen zoals insulineresistentie, microvasculaire pathologieën (zoals diabetische retinopathie en nefropathie) en cardiovasculaire ziekten. Een ernstige ontregeling van wit vetweefsel zorgt daarnaast voor een verhoogde productie van reactieve zuurstofradicalen (ROS) en een afname in glyoxalase-1 activiteit (GLO1, het enzymatisch detoxificatie systeem van methylglyoxaal). Beide dragen bij aan verhoogde dicarbonylstress. In dit proefschrift ligt een sterke focus op methylglyoxaal (MGO), de meeste reactieve dicarbonylverbinding die geassocieerd is met de ontwikkeling van diabetes.

In hoofdstuk 2 hebben we vastgesteld dat postprandiale plasma MGO-, GO- en 3-DG concentraties aanzienlijk hoger waren bij mannen met abdominale obesitas in vergelijking met slankere mannen. Deze bevinding bevestigt eerder verzamelde gegevens van (morbide) obese vrouwen met diabetes. We beschreven dat grote hoeveelheden dicarbonyls herhaaldelijk worden geproduceerd tijdens postprandiale glucosepieken en verhoogd zijn bij obese mannen. Deze toename in dicarbonyls kan bijdragen aan complicaties die verband houden met obesitas; net daarom zijn preventieve interventies in deze populatie aangewezen.
Met uitzondering van Nε-(carboxymethyl)lysine (CML) waren de concentraties van AGE's in plasma en in de huid (gemeten met autofluorescentie) niet verschillend tussen slanke en obese individuen, wat zou kunnen wijzen op een beperkt effect op circulerende AGE's in deze fase van de pathologie. Dit sluit echter niet uit dat er geen AGE-ophoping is in weefsels van obese individuen. De afname van plasma CML bij obese mannen (hoofdstuk 2) is in overeenstemming met de opname van CML in vetweefsel bij obesitas, zoals eerder beschreven.

Gewichtsverlies

Onze studie in **hoofdstuk 2** toonde aan dat gewichtsverlies in een populatie met abdominale obesitas de dicarbonylstress verminderde, vooral de postprandiale niveaus. Plasma dicarbonyls zakken tot het niveau van slanke individuen. Op dezelfde wijze werd ook proteïnegebonden Nε-(carboxyethyl)lysine (CEL) verminderd, maar andere AGE's werden niet beïnvloed door de interventie.
Onze studie kon bevestigen dat het effect op glucosewaarden een groot deel van de verlaging van dicarbonyls verklaart. Het is ook mogelijk dat calorierestrictie het glyoxalase-systeem kan activeren, mogelijk via activatie van Nrf2, hoewel we geen aanwijzingen vonden voor dit onderliggende mechanisme. Een toename van ketolichamen zoals acetoacetaat en β-hydroxyboterzuur, die MGO kunnen opruimen, zijn andere mogelijke redenen voor de afname in dicarbonyls.

Fysieke activiteit

De resultaten van fysieke activiteit op dicarbonyls en AGE's blijken complexer om te interpreteren. Een vermindering van sedentaire tijd en een toename van algemene fysieke activiteit zijn sterk geassocieerd gebleken met insulinegevoeligheid, terwijl verhoogde lichamelijke oefening meestal wordt geassocieerd met vasculaire gezondheid.
Echter, menselijke studies die het effect van lichaamsbeweging op dicarbonylstress of AGE's beoordelen, hebben tegenstrijdige resultaten opgeleverd. Specifieke intensiteit en duur van de oefeningen, naast populatieverschillen, zijn een mogelijke verklaring voor de waargenomen tegenstrijdigheden. In **hoofdstuk 3** toonden we aan dat actieve individuen hogere niveaus van plasma CML hadden dan sedentaire individuen. Bovendien vonden we een positieve associatie tussen CML en VO_2-max. Het is mogelijk dat lichaamsbeweging weefselherstel bevordert en de afbraak van crosslinks in de vaatwand stimuleert. Dit kan leiden tot hogere niveaus van circulerende (proteïne-gebonden) AGE's. Daarnaast kunnen een hoger metabolisme en een hogere energie-inname, resulterend in hogere AGE niveaus via vorming en inname, ook een verklaring zijn.
Bovendien toonden we aan dat intervaltraining met hoge intensiteit (HIIT) geen materiële veranderingen teweegbracht in plasma dicarbonyl of AGE concentraties.

Flavonoïden

In **hoofdstuk 4** hebben we aangetoond dat de flavonoïde quercetine leidde tot een verlaging van plasma MGO bij (pre)hypertensieve volwassenen. Er werden geen significante effecten waargenomen voor AGE's. De toediening van epicatechine resulteerde niet in significante dalingen van dicarbonyls of AGE's. Deze bevindingen

waren in lijn met eerder experimenteel werk dat aantoonde dat quercetine circulerend MGO kon verlagen. Eerdere bevindingen dat quercetine of epicatechine ook de vorming van AGE's remmen, konden we niet reproduceren. Quercetine oefent waarschijnlijk zijn verlagende effect op MGO-niveaus uit via het biochemisch opruimen aangezien we geen effecten op glucosewaarden of glyoxalase-1 activiteit vonden. Meerdere experimentele studies hebben ongeconjugeerde vormen van flavonoïden of suprafysiologische doseringen gebruikt, wat de verschillen met experimentele modellen kan verklaren. Bovendien vonden we in dezelfde studie dat suppletie met quercetine significant leidde tot een afname van sE-selectine, IL-1β en een z-score van ontstekingsmarkers. Helaas ontbrak het ons aan statistische kracht om een mogelijk effect van MGO-vermindering door quercetine op de ontstekingsmarkers te bestuderen.

Pyridoxamine

Pyridoxamine is een B6-vitameer die in experimentele studies in staat bleek om MGO-concentraties te verlagen en de vorming van AGE's te remmen. Humane interventiestudie die de rol van pyridoxamine in de preventie van metabole en vasculaire disfunctie onderzoeken, ontbraken tot op heden. In ons klinisch onderzoek, beschreven in **hoofdstuk 5 tot en met 8**, hebben we het effect van pyridoxamine bestudeerd bij individuen met overgewicht.

In **hoofdstuk 6** hebben we een methode geoptimaliseerd en gevalideerd voor de kwantificering van vitamine B6 in plasma en urine met behulp van UPLC-MS/MS. Na een metabolisatie-experiment hebben we aangetoond dat pyridoxamine supplementen mogelijk een voordeel bieden ten opzichte van de huidige B6-supplementen. We toonden aan dat pyridoxamine-inname resulteerde in hoge niveaus van pyridoxal 5'-fosfaat, de biologisch actieve vorm van vitamine B6. De vorming van pyridoxine was minimaal. Pyridoxine is momenteel het meest gebruikte B6-supplement en is de belangrijkste oorzaak van vitamine B6-toxiciteit. Hoge niveaus van pyridoxine zijn neurotoxisch en deze vorm van B6 is ook een competitieve remmer van pyridoxaal 5'-fosfaat. Gecombineerd met de gegevens van hoofdstuk 7 kan dit pyridoxamine aantrekkelijker maken als supplement voor zowel de behandeling van metabole ziekten als vitamine B6-tekort. Vanwege de snelle metabolisatie moet worden opgemerkt dat de drie dagelijkse dosissen resulteerden in een meer constante plasmaconcentratie gedurende de dag.

In **hoofdstuk 7** toonden we aan dat pyridoxamine effectief de plasmaniveaus van MGO en MGO-afgeleide AGE Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) verminderde. De afname in MGO was dosisafhankelijk, waarbij de hoge dosering van pyridoxamine leidde tot een 9% vermindering van plasma MGO vergeleken met de baselinewaarden. Hoewel deze afname bescheiden lijkt, is deze vergelijkbaar met

eerdere studies (onder andere gewichtsverlies en quercetine). Bovendien observeerden we een niet-significante afname van andere AGE's. Pyridoxamine toonde echter geen verbetering van insulinegevoeligheid of vaatfunctie in onze studie. Op basis van onze eerdere dierproeven verwachtten we enig positief effect op glucosemetabolisme, insulinegevoeligheid of microvasculaire functie te vinden. Een mogelijke verklaring voor deze nulresultaten is dat muizen gevoed met HFD een extremer model zijn en de dosering van pyridoxamine in onze dierenstudie vijf keer hoger was in vergelijking met de hoogste dosering in onze klinische studie. Het behandeleffect in onze overgewichtspopulatie was waarschijnlijk te laag om functionele uitkomsten te beïnvloeden. Wel opmerkelijk was de significante afname van plasmamarkers van endotheel-disfunctie, met name oplosbare vasculaire celadhesiemolecuul-1 (sVCAM-1) en oplosbaar intercellulair adhesiemolecuul-1 (sICAM-1).
In hoofdstuk 8 toonden we aan dat arteriële stijfheid niet werd beïnvloed door pyridoxamine behandeling gedurende de periode van acht weken. Arteriële stijfheid werd grondig onderzocht met bepaling van onder andere carotis distensibiliteit, polsgolfsnelheid en de intima-media-dikte van de carotis. Het effect van pyridoxamine op MGO en AGE's was waarschijnlijk te klein om effecten op arteriële stijfheid te veroorzaken binnen de termijn van de studie.

Conclusie

Zowel gewichtsverlies, pyridoxamine, als quercetine laten ongeveer een 10% daling zien in plasma MGO, vergezeld van positieve effecten op markers van ontsteking en endotheel-disfunctie. Pyridoxamine toont ook een vermindering van AGE's, echter zonder effecten op insulinegevoeligheid of vaatfunctie. Fysieke activiteit laat in onze studie geen verbetering zien in relatie tot dicarbonyls of AGE's.
Associaties tussen dicarbonyls, AGE's en metabole ziekten zijn overvloedig beschreven in de literatuur. Op basis van de studies beschreven in dit proefschrift weten we dat het mogelijk is om effectief MGO te verlagen bij ogenschijnlijk gezonde individuen met overgewicht. De vraag blijft wat het behandeleffect van deze interventies op methylglyoxaal moet zijn om op de lange termijn een verbetering of preventie van het ziekteproces te kunnen opleveren.

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Curriculum vitae

Mathias Van den Eynde was born on November 23rd 1990 in Edegem, Belgium. He graduated from secondary school (Sint-Lievenscollege, Antwerp) in 2008. The same year he started his studies in Biomedical Sciences at the University of Antwerp. During his master he specialized in Molecular Imaging as a major and Molecular and Cellular Medicine as a minor, with internships at the laboratory of Cell Biology and Histology, and the Molecular Imaging Center Antwerp. During this last internship he evaluated a radioactively labelled urokinase inhibitor as an imaging biomarker in cancer models. In 2014 he graduated with distinction from the University of Antwerp. From 2015 to 2020 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. Boy Houben he investigated intervention strategies in the glycation pathway, as presented in this thesis. During this time, his main focus was on performing a clinical trial with pyridoxamine. In may 2021 he started working as a teacher at Zuyd University of Applied Sciences.

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