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Interplay of methylglyoxal and immune cells

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Interplay of methylglyoxal and immune cells: implications for type 2 diabetes?

Xiaodi Zhang

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Interplay of methylglyoxal and immune cells: implications for type 2 diabetes?

DISSERTATION

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Chapter 1

General introduction

Type 2 diabetes, inflammation, and diabetic vascular complications

People suffer from type 2 diabetes (T2D) are at high risk of developing microvascular (retinopathy, nephropathy, neuropathy) and macrovascular (peripheral artery, coronary artery, and cerebrovascular) diseases[1]. Cardiovascular disease is the leading cause of mortality in T2D[2,3]. It has been previously shown that both hyperglycaemia and obesity are in close association with the morbidity and mortality of diabetic micro- and macrovascular complications[3-8]. Improving glycaemic control and weight loss can reduce the burden of diabetes, however, a substantial residual cardiovascular risk remains in people with T2D[1,3]. Low-grade inflammation has been considered to play an essential role in this residual risk of cardiovascular disease[9-11]. However, the underlying mechanism is yet not well understood.

Increasing evidence has shown a link between innate immune cells and chronic inflammation that hallmarks T2D and its vascular complications[12-16]. Activated immune cells are often accompanied by an elevated glycolysis for the energy demand[17]. The dicarbonyl compound methylglyoxal (MGO), generated as a by-product of glycolysis, has been suggested to modulate immune cell activation[18-20]. In addition, elevated levels of MGO are also commonly observed in diabetes and in inflamed tissues such as carotid atherosclerotic plaques and obese adipose tissue[21-24]. This thesis will further focus on the interaction between MGO and innate immune cells, in relation to obesity and T2D. The effects of MGO on immune cells is extensively described in Chapter 2, and therefore this current chapter will mainly introduce the alterations of immune cells and MGO in low-grade inflammation, as well as their potential role in T2D and its complications.

Immune cells and their activation

Inflammation is a critical process that defends against pathogens and also repairs tissue damage and regains tissue homeostasis[25]. Inflammatory response involves innate immune cell (neutrophils, monocytes, and macrophages) recruitment and activation. Under pathological conditions, crosstalk of immune regulation with abnormal metabolism results in chronic sterile inflammation.

Dysregulation of immune cells in this chronic inflammation has been suggested to play a role in the development of metabolic diseases[26].

Immune cells

Immune cells develop from stem cells within the bone marrow and are an essential part of the immune system. Cells involved in the innate immunity include neutrophils, monocytes, macrophages, dendritic cells, and natural killer (NK) cells, which provide immediate host defence. Adaptive immune cells consist of T and B lymphocytes, which precisely respond to specific antigens and develop long-lasting memory[27]. This thesis mainly focuses on neutrophils, monocytes, and macrophages, as they are highly sensitive to hyperglycaemia and are involved in glucose-induced inflammation[12,14]. In the initial pro-inflammatory phase, neutrophils and monocytes are released from the bone marrow into the circulation, in response to CXCR2 ligands and CCR2, respectively[28,29]. Then, these cells are recruited and activated at the site of infection to eradicate pathogens[26,27,30]. This pro-inflammatory response is usually followed by a resolution process, during which neutrophils undergo apoptosis, while macrophages are switched from pro- (M1) towards anti-inflammatory (M2) phenotype for tissue repair and remodelling. Under hyperglycaemic conditions, this resolution process is disrupted, at which point M1 macrophages are continuously activated and failed to switch to M2 phenotype, resulting in persistent inflammation[26].

Immunometabolism

Specific alterations in metabolic pathways are essential for fulfilling the needs of cell survival or growth. There are six key metabolic pathways, including glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate pathway (PPP), fatty acid oxidation, fatty acid synthesis, and amino acid synthesis[31]. Macrophage immunometabolism has been extensively reviewed previously[17,31,32]. Briefly, upon pro-inflammatory activation, macrophages switch from oxidative phosphorylation to glycolysis. PPP is also elevated and leads to production of nicotinamide adenine dinucleotide phosphate (NADPH), which is used for the generation of reactive oxygen species (ROS) and nitric oxide (NO). TCA cycle is broken in two places, after citrate and after succinate, resulting in elevated fatty acid synthesis and activated hypoxia-inducible factor 1α (HIF1α), respectively[17,31,32]. HIF1α is known to promote glycolysis and inflammatory signalling, which leads to increased secretion of cytokines such as interleukin-

1β[33]. Similar to macrophages, activated neutrophils also undergo glycolysis and produce NADPH in the PPP. Whereas this increased PPP activity not only promotes ROS production but also supports NOX2 function, which subsequently promotes antimicrobial functions of neutrophils as well as eventual death by neutrophil extracellular trap formation (NETosis). Although elevated PPP is also observed in activated macrophages, NETosis is not shown to be a feature of these cells. In addition, neutrophils contain very few mitochondria and therefore are less dependent on the TCA cycle for ATP production[34-36].

Trained immunity

Immune memory is an essential hallmark of the adaptive immune system. However, in recent years, increasing evidence has indicated that activation of innate immune cells as well as myeloid progenitor cells can also result in enhanced responsiveness to subsequent triggers[37-39]. Trained immunity is defined as the long-term functional reprogramming of innate immune cells, which is characterized by epigenetic modifications and metabolic reprogramming. Trained immunity can be induced by exogenous or endogenous insults, which leads to an altered response to subsequent time-delayed heterologous stimulation[37]. Innate immune cells that are able to build trained immunity or comparable adaptive memory characteristics include monocytes, macrophages, dendritic cells, and NK cells, however, without neutrophils, due to their high refreshment rate of these cells[38,40]. It has been currently discovered that two key epigenetic modifications are involved in the induction of trained immunity: 1. the acquisition of histone 3 lysine 27 acetylation (H3K27ac) modifications at distal enhancers that are carrying with histone 3 lysine 4 methylation (H3K4me1) modifications, and 2. the consolidation of histone 3 lysine 4 trimethylation (H3K4me3) modifications at the promoters of stimulated genes[37]. During the epigenetic reprogramming, stimulation of innate immune cells leads to unfolding of chromatin and promotes transcription and expression of pro-inflammatory factors. After cessation of the stimulation, cells return to the resting state while modifications are only partially removed, which allows a faster and enhanced recruitment of transcription factors and gene expression in response to a secondary heterologous stimulation[37] (Figure 1.1). In addition, reprogramming of metabolic pathways in trained immunity may also be involved in the regulation of epigenetic programs by providing intermediate metabolites, as substrates for epigenetic enzymes, or as co-activators or co-repressors of epigenetic writers or erasers[40,41].

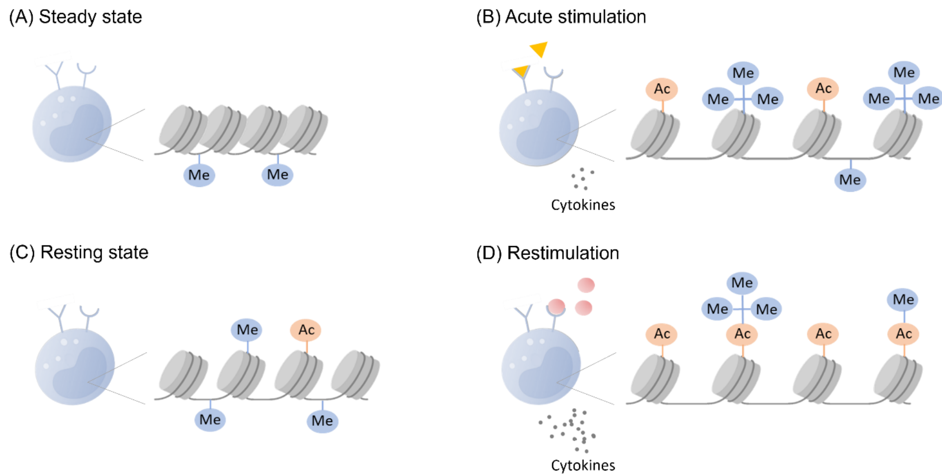

Figure 1.1 Epigenetic reprogramming during the induction of trained immunity. (**A**) Steady state: little gene expression, high DNA methylation; (**B**) acute stimulation: active transcription, low DNA methylation; (**C**) resting state: little gene expression, mild DNA methylation; (**D**) restimulation: enhanced gene expression, low DNA methylation. Figure adapted from[37].

Immune response in diabetes and its complications

Elevated neutrophil-lymphocyte ratio is a common hallmark of T2D and predicts disease progression[42,43]. Hyperglycaemia activates neutrophils, causing increased production of ROS[44,45], subsequent increased NETosis[46,47], and upregulates cytokine signalling and cell adhesion pathways[16,48]. Within atherosclerotic plaques, release of NETs promotes the activation of M1 macrophages via NLRP3-dependent inflammasome activation, leading to elevated production of IL-1β and IL-18, which in turn contributes to NETosis. Hyperactivated neutrophils can induce vascular smooth muscle cell death, resulting in plaque destabilization[16]. In addition to atherosclerosis, constitutive neutrophil activity is also linked to other diabetic complications, including diabetic kidney disease, retinopathy, peripheral neuropathy, and foot ulcers[26,49-52].

Dysregulation of monocytes/macrophages has also been implicated in diabetes-related pathological conditions, such as adipose tissue dysfunction, atherosclerosis, non-healing wounds, and kidney disease[26]. Under hyperglycaemic conditions, upregulation of glycolysis and polarisation of M1 macrophage phenotype are thought to link with epigenetic changes[53]. Glycolytic intermediate such as pyruvate kinase M2 can interact with histone deacetylases

and cause chromatin remodelling, which promotes pro-inflammatory responses of macrophages[53,54]. Epigenetic alterations on TLR4 promoter influence responsiveness of diabetic macrophages to TLR4 stimulation and inhibit tissue repair[55]. Studies in human and animal models demonstrate that the epigenetic changes persist even after significant weight loss and achieving glucose control[26,56,57]. In line with these findings, hyperglycaemia has been shown to induce trained immunity in bone marrow progenitor cells via persistent epigenetic modifications, which persists after differentiation into macrophages. This hyperglycemia-induced trained immunity phenotype is further identified in circulating leukocytes from people with T2D, which may explain the remaining higher cardiovascular risk in diabetes even after glucose control[58].

Methylglyoxal in low-grade inflammation

Elevated MGO levels are observed under hyperglycaemic conditions, and are linked to T2D and its complications[59]. This accumulation of MGO may play an important role in the glucose-induced inflammation as well as the etiology of these metabolic diseases. Therefore, pathways involved in the metabolism of MGO and the potential effects of MGO in T2D will be discussed.

Metabolism of methylglyoxal

The formation and detoxification of MGO has been reviewed in detail in Chapter 2. In short, MGO is mainly produced from the non-enzymatic degradation of glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone-phosphate (DHAP), during glycolysis. Smaller quantities of MGO are formed from the glucose autoxidation, degradation of glycated proteins, and metabolism of acetone, aminoacetone, and threonine, as well as from food and drinks[59-61]. The detoxification of MGO mainly involves the glyoxalase system, which is composed of two major enzymes: glyoxalase I and II (GLO1 and GLO2). In this pathway, hemithioacetal, the spontaneously formed product of glutathione and MGO, is firstly catalysed by GLO1 into S-d-lactoylglutathione, which is subsequently hydrolyzed by GLO2 in D-lactate[59,60]. In addition, MGO is the major precursor of endogenously formed advanced glycation endproducts (AGEs), which are generated via modification of the amino acids[62]. The irreversible reaction of MGO with arginine mainly generates hydroimidazolones (MG-Hs), over 90% of which is MG-H1, and reaction with lysine residues mainly forms N^{ε}-(carboxyethyl)lysine (CEL)[59,63]. Apart from the modifications on amino

acids, MGO can also modify nucleic acids and lead to formation of nucleotide AGEs, mainly N(2)-carboxyethyl-2′-deoxyguanosine (CEdG) and 3-(2-deoxyribosyl)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b]purin-9(8)one (MGdG)[64].

Abnormal metabolism of methylglyoxal in diabetes and its complications

Higher plasma levels of MGO and MGO derived AGEs are commonly shown in people with diabetes, as a consequence of elevated glucose concentrations[23,65,66]. In agreement, decreased expression of GLO1 is also observed under hyperglycaemic and inflammatory conditions[59]. Experimental studies *In-vivo* have demonstrated a role of MGO in diabetes-related endothelial dysfunction. Administration of MGO in rat induces diabetes-like microvascular changes, including impaired vasodilation, degenerative changes in cutaneous microvessels with loss of endothelial cells, basement membrane thickening, luminal occlusion, as well as increased oxidative stress[67,68]. In line with these findings, GLO1 overexpression reduces diabetes-induced impairment of vasorelaxation and prevents diabetes-induced expression of endothelial dysfunction markers vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) in rat mesenteric arteries[69]. Endothelial dysfunction and expression of VCAM-1 and ICAM-1 is linked with activation of transcription factor nuclear factor-κB (NF-κB) in diabetes. A potential pathway for these vascular changes that induced by MGO may be the changes of epigenetics. Set7 induces modification of H3K4m1 on the promoter of the RelA gene encoding for the NF-κB p65[59]. Overexpression of GLO1 prevents hyperglycaemia-induced persistent increases in Set7-mediated histone methylation and NF-κB p65 gene expression, indicating a significant effect of MGO in epigenetics[59,70]. This ability of MGO to modify DNA may explain the failure in reducing cardiovascular risk in people with T2D due to the legacy effect of hyperglycaemia.

Objectives and outline of this thesis

The major objective of this thesis is to investigate the interactions between MGO and innate immune cells in relation to type 2 diabetes. This thesis will mainly focus on the formation of MGO in humans and in mice during a glucose tolerance test, and the effects of excess MGO on immune cells. Figure 1.2 illustrates a schematic overview of the studies included in this thesis.

In **Chapter 2**, we reported a review about the general formation and metabolism of MGO, as well as the anti-microbial effects of MGO and the link between MGO and immune cell activation, as potential mediator during host defense.

In **Chapter 3**, we investigated *in vivo* whether postprandial MGO formation directly originates from exogenous glucose during a glucose tolerance test in human plasma and in mouse tissues, using universally labelled D(+)^{13}C glucose. In addition, we investigated *in vitro* whether the increased plasma MGO concentration leads to a fast formation of MGO-derived AGEs.

In **Chapter 4**, we evaluated MGO concentrations in circulating cells and investigated in *vivo* whether MGO formation in these cells during a glucose tolerance test originates from exogenous glucose, and whether obesity affects glucose-derived MGO formation.

In **Chapter 5**, we assessed *in vivo* the contribution of glycolysis to the exogenous glucose-derived MGO formation in mouse plasma, circulating cells, and tissues during a glucose tolerance test.

In **Chapter 6**, we investigated whether MGO directly affects immune cell counts and activation *in vivo*. In addition, since MGO has the ability to modify DNA and inducing epigenetic changes, we also investigated the potential effects of MGO on trained immunity.

In **Chapter 7**, we investigated the associations of plasma MGO concentrations prior to and after an oral glucose tolerance test with circulating immune cell counts and activation, in a population-based cohort study (The Maastricht Study).

In **Chapter 8**, findings of *in vitro* experiments, *in vivo* studies, and the cohort study from this thesis are summarized and their clinical relevance is discussed.

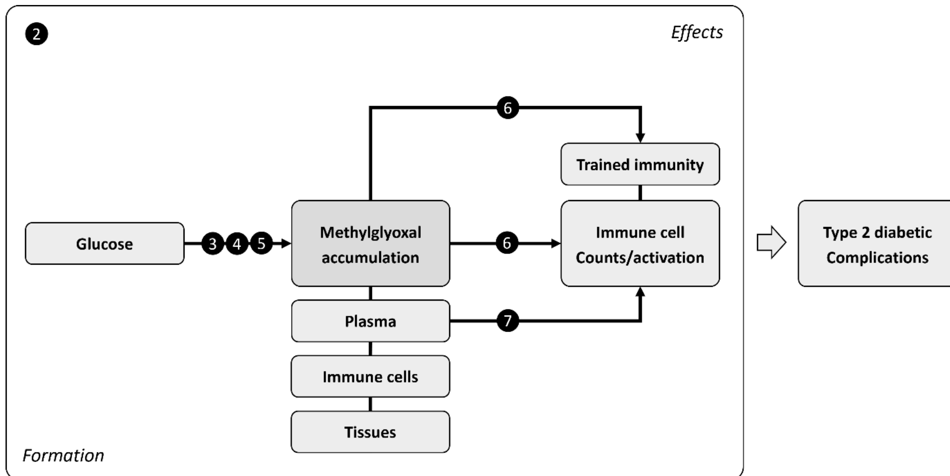

Figure 1.2 Schematic overview of the studies included in this thesis. Numbers indicate thesis chapters. Solid arrows represent relations that we hypothesized and investigated. Chapter 2 is a review article about the formation of MGO and the link with inflammatory activation of immune cells, in relation to diseases.

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Chapter 2

Immunometabolism and the modulation of immune responses and host defense: A role for methylglyoxal?

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Abstract

The immune system plays an essential role in protecting the body against pathogens. Immune cells are activated during infections, resulting in a metabolic shift from oxidative phosphorylation to glycolysis. During glycolysis, methylglyoxal (MGO) can be formed as a by-product. As a highly reactive dicarbonyl compound, MGO can rapidly react with proteins to form advanced glycation end products (AGEs). MGO and MGO-derived AGEs have been implicated in the development of insulin resistance, type 2 diabetes and its complications and several other age-related inflammatory diseases. MGO has been found in adipose tissue, atherosclerosis plaques and inflamed livers. Aside from the potential harmful role of MGO, there are studies showing beneficial effects of MGO as a defense mechanism during infections and diseases. In this review, we summarize anti-microbial effects of MGO and the link between MGO and immune cell activation, as potential mediator during host defense.

Introduction

The immune system is crucial for host defense during infections, but also for tissue homeostasis and wound healing[1,2]. The immune system consists of an innate and an adaptive part. Innate immune cells are mainly composed of phagocytes such as neutrophils, monocytes, macrophages, dendritic cells (DCs) and innate lymphoid cells, including natural killer (NK) cells. Phagocytes respond rapidly and non-specifically to pathogens via the engagement of pattern recognition receptors. Their main effector function is phagocytosis and killing of pathogens in order to stop infection. In addition, innate immune cells produce cytokines to cause acute inflammation and can activate the adaptive immune system[3]. Adaptive immune cells include T and B lymphocytes, which have the ability to recognize specific antigens in responding to the pathogens or infected cells[3]. B-cell activation leads to the production of antibodies with several anti-microbial functions. T-cells can be subdivided into CD8+ cytotoxic T cells that kill virally infected and transformed cells, and CD4+ helper T cells, which produce cytokines to regulate immune responses, having diverse effects on almost all other immune cells. An important feature of adaptive immunity is their specificity to specific antigens, and their ability to develop long-lasting immunological memory[3,4].

Immune cell activation is often associated with shifts in cellular energy metabolism from oxidative phosphorylation (OXPHOS) to glycolysis to facilitate the fast requirement of energy. Although OXPHOS is more efficient making ATP (38 ATP molecules from 1 molecule of glucose) than glycolysis (2 ATP molecules from 1 molecule of glucose), glycolysis operates much faster. Glycolysis can be rapidly activated by the catalysis of a series of enzymes, while inducing OXPHOS requires more complex process, involving the biogenesis of mitochondria. Moreover, glycolysis also supports other metabolic pathways by providing biosynthetic intermediates[5]. Enhanced glycolysis has been found in almost all activated immune cells including macrophages, DCs, natural killer cells, effector T cells, and B cells[5], and affects a plethora of cellular functions, such as phagocytosis and inflammatory cytokine expression in macrophages, antigen presentation in DCs, and effector cytokine production in T cells[5-7].

In addition to these cellular switches to glycolysis upon inflammatory activation, immune cell activation is also linked with the development of peripheral insulin resistance[8]. Insulin is produced in the beta cells of the pancreas and promotes

cellular glucose uptake in peripheral tissues by inducing the translocation of the glucose transporter GLUT4 to the cell surface[9]. Moreover, insulin stimulates an anabolic state by stimulating glucose storage in the form of glycogen in the liver and muscle as well as promoting fat storage via increased lipogenesis[9,10]. Under the condition of insulin resistance, the responsiveness to circulating insulin is reduced, hampering GLUT4 translocation and leading to a reduction of glucose uptake in the target tissues, and an increase in blood[10]. Insulin resistance is mainly linked with the presence of chronic inflammation that accompanies obesity and is considered a major risk factor for developing type 2 diabetes. However, the link between inflammation and insulin resistance is possibly an evolutionary adaptive trait and activated immune cells may contribute to the induction of insulin resistance[8,11,12]. During infection, the body enters into a state of temporary insulin resistance. Insulin resistance decreases glucose uptake in insulin-dependent tissues such as skeletal muscles and the liver[13], and this accommodate the extremely high energy demands of the immune system during infection[8]. Monocytes and lymphocytes were shown to express high levels of GLUT1 and 3, but not of the insulin-sensitive GLUT4, suggesting that these cells can take up glucose even under conditions of insulin resistance[14]. These mechanisms may now be maladaptive in times of constant nutrient availability, driving chronic metabolic stress[12].

During glycolysis, the highly reactive dicarbonyl compound methylglyoxal (MGO), is formed, presumably as a by-product[15]. MGO rapidly reacts with proteins, leading to the production of advanced glycation endproducts (AGEs). Studies have shown both MGO and MGO-derived AGEs accumulate in immune cells in inflamed tissues, such as obese adipose tissue[16,17], atherosclerotic plaques[18,19] and inflamed liver[20,21]. MGO has been linked to insulin resistance, cardiometabolic diseases and its complications[15,22], and is thought to be a predictor of hypertension in type 2 diabetic patients, which is also a major risk factor for chronic kidney disease[23,24]. Moreover, MGO may also be a specific target of cardiovascular disease as higher plasma MGO levels are associated with total cardiovascular diseases and with all-cause mortality, myocardial infarction, and amputations in individuals with type 2 diabetes, while higher levels of other dicarbonyl compounds such as glyoxal and 3-deoxyglucosone in plasma are not[25]. However, in immune cells, it is less clear whether MGO is merely a toxic by-product of enhanced glycolysis, or whether it plays an active role in immune cell effector functions. In this review, we will discuss how MGO affects immune cell functions in relation to infection and disease.

Methylglyoxal

Formation of methylglyoxal

MGO has been discovered in biological systems hundred years ago[26]. MGO is present in all cells, as well as body fluids in human and animals[27-29]. There are different pathways of MGO formation (Figure 2.1)[15] and among these pathways, the non-enzymatic degradation of the glucotrioses glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), which are formed during glycolysis, is thought to be the major source. Although MGO is only produced as a small fraction in the glycolytic flux, glycolysis contributes about 90% to the total amount of MGO[15,27,30]. Other endogenous sources of MGO include auto-oxidation of glucose and degradation of glycated proteins[31], oxidation of aminoacetone in threonine catabolism[32], oxidation of acetone in ketone body metabolism[33], and lipid peroxidation[15,34].

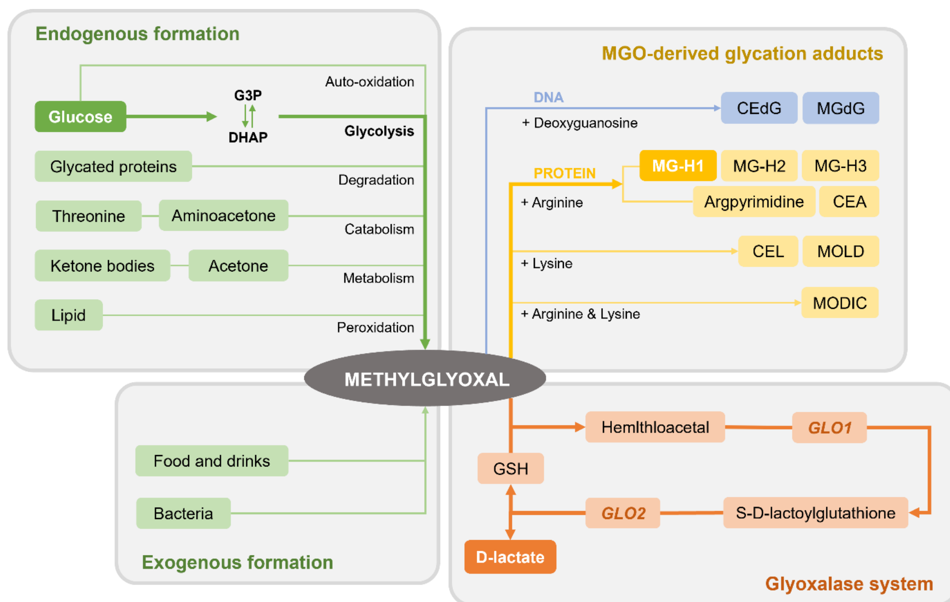

Figure 2.1 Formation and detoxification of methylglyoxal (MGO), and MGO-derived glycation adducts. G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; CEdG, N(2)-carboxyethyl-2'-deoxyguanosine; MGdG, 3-(2-deoxyribosyl)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b]purin-9(8)one; MG-Hs (MG-H1, MG-H2, MG-H3), MGO-derived hydroimidazolones; CEA, carboxyethyl arginine; CEL, N^{ε}-carboxyethyl-lysine; MOLD, lysine-derived 4-methylimidazolium crosslink; MODIC, arginine–lysine-derived crosslink; GSH, glutathione; GLO1, glyoxalase I; GLO2, glyoxalase II.

Exogenous sources of MGO are mainly from diet, where MGO levels are strongly affected by sugar and lipid content, heating, the formation of Maillard reaction products, and microbial fermentation[28,35]. Some food and drinks contain high concentrations of MGO, such as snacks and nuts, cookies/bakery, meat and fish, coffee, and dairy/soy drink[36]. MGO contents are extremely high in Manuka honey[36,37]. Interestingly, Manuka honey has been described to have anti-bacterial properties, which are attributed to the presence of MGO[38]. Based on estimations of food intake in a large cohort study, Maasen et al. calculated a daily dietary intake of MGO of 3.6 mg/day and approximately 25% of the daily intake is from coffee[39]. This amount of the daily intake of MGO is relatively low compared to the total MGO levels in the body (<0.1%). More than 80% of MGO is degraded within 8 hours during digestion before absorption, potentially via chemical reactions with amino acids such as lysine and arginine in the gastrointestinal tract and the detoxification effects of glyoxalase system from epithelial cells in the small intestine[40,41]. Short-term (within 4 weeks) high MGO intake from Manuka honey does not affect MGO-derived AGEs accumulation in serum[42], while long-term (3 months) higher intake of dietary MGO is associated with higher plasma MGO concentrations and skin autofluorescence (a biomarker for skin AGEs) in humans[39]. The exact effects of dietary MGO on health remain, however, unclear. Knowledge of MGO concentrations and distribution in human tissues are limited. Aside from endogenous and dietary sources, bacteria may also be a source of MGO. Different from mammalian cells, MGO formation in Escherichia coli cells originates mainly from enzymatic degradation of DHAP in glycolysis, where MGO synthase is involved[43].

Detoxification of methylglyoxal

To protect cells from the toxicity of MGO, many pathways have evolved that contribute to the detoxification of MGO. In mammals, more than 99% of MGO formed in the body is metabolized to D-lactate by the glyoxalase system[44]. The glyoxalase system is present in the cytosol of all cells and is composed of glutathione (GSH) and two enzymes: glyoxalase I and II (GLO1 and GLO2). In this pathway, GSH reacts with MGO spontaneously to generate hemithioacetal. Under the catalysis of the rate-limiting enzyme GLO1, hemithioacetal is then metabolized to S-D-lactoylglutathione, which is hydrolyzed to D-lactate via the action of GLO2. During this GLO2-catalyzed reaction, GSH is regenerated and is used for the next cycle[45,46].

Within the glyoxalase system, GLO1 is the rate-limiting step. The mechanisms regulating GLO1 expression and activity are complex, but under inflammatory conditions, a decrease of GLO1 expression was observed in human carotid atherosclerotic plaques, which coincided with an accumulation of plaque MGO and AGEs[47]. In line, experimentally induced inflammation in healthy individuals transiently reduced GLO1 expression in leukocytes. This decrease in GLO1 expression did not lead to a detectable increase in plasma MGO[48]. Moreover, GLO1 expression and activity were found to be reduced by TNF in vitro using the monocytic cell line U937, which led to increased MGO and AGE formation. Increased levels of MGO by a downregulation of GLO1 under inflammatory conditions may lead to harmful effects including the induction of cellular dysfunction and cell death[47]. Indeed, inducing GLO1 in overweight and obese subjects showed beneficial effects, including decreased plasma MGO levels, reduced vascular inflammation, and improved glycemic control[49]. In line with these findings, GLO1 overexpression in a diabetic rat model prevented vascular intracellular glycation and improved endothelial dysfunction and early renal dysfunction[50]. Activating the glyoxalase system may thus be beneficial to treat the development of diabetes and its vascular complications by reducing MGO levels.

In addition to the glyoxalase system, other enzymes, such as aldehyde dehydrogenases and aldoketo reductases, can also metabolize MGO to form pyruvate or the metabolite hydroxyacetone respectively[51,52]. Interestingly, the ketone body acetoacetate is found not only to contribute to the formation of MGO, but also can scavenge MGO by a non-enzymatic conversion to 3-hydroxyhexane-2,5-dione (3-HHD)[53,54]. Furthermore, Lee et al. reported that the protein deglycase DJ-1 has glyoxalase activity, converting MGO directly to lactic acid without involvement of GSH[55]; it can also repair MGO-glycated amino acids and proteins, and release repaired proteins and lactate[56]. Similar to DJ-1, glyoxalase III (GLO3) can also detoxify MGO directly to D-lactate independent of GSH[57]. However, GLO3 seems to be present specifically in bacteria.

Methylglyoxal-derived glycation adducts

As a highly reactive dicarbonyl compound, MGO rapidly binds to proteins or nucleic acids to form glycation adducts, which can be classified as MGO-AGEs and MGO-derived DNA adducts. The irreversible reaction between MGO and arginine residues leads to the formation of MGO-derived AGEs (mainly hydroimidazolones: MG-H1, MG-H2, MG-H3), of which MG-H1 is the major

isomer under physiological conditions (>90% of the formed adducts). Minor MGO-derived AGEs of arginine residues are argpyrimidine and carboxyethylarginine (CEA). The reaction between MGO and lysine residues can generate N^{ε}-(carboxyethyl)lysine (CEL). In addition, there are also cross-link proteins formed during the reactions, such as lysine-derived 4-methylimidazolium crosslink (MOLD) and arginine–lysine-derived crosslink (MODIC)[53,58,59]. The levels of these MGO-derived AGE residues in cellular proteins are much higher than in plasma proteins[58], suggesting that the majority of MGO is formed within cells and reacts immediately with cellular proteins.

MGO-derived AGEs are increased in people with diabetes, as a result of hyperglycemia[60]. In rat model of diabetes, AGEs were found to accumulate in renal glomeruli, retina, peripheral nerve, and plasma, feeding the thought that AGEs are linked to the development of diabetic complications[58]. In addition, MGO-derived AGEs accumulate in human carotid atherosclerotic plaques[18], and in astrocytes from brain samples of patients with multiple sclerosis, an auto-immune disease targeting the central nervous system[61]. MGO can modify multiple proteins including albumin, collagen, haemoglobin and lens proteins[29], leading to the formation of MG-H1 on these target protein, cellular dysfunction[62-64] and to the production of pro-inflammatory cytokines[65,66]. These effects may be, mediated at least partially, by binding of AGE-modified proteins to cell surface receptors[67]. A recent study found that MG-H1 is a ligand for the receptor for AGEs (RAGE)[68]. However, the concentration of MG-H1-modified proteins in human plasma is about 30 times higher than RAGE protein on human monocytes[45,69,70]. Although there are many studies showing that RAGE is linked to diseases such as diabetic chronic kidney disease, multiple sclerosis, and inflammation[61,71,72], it is thus unlikely that MG-H1 is a physiological relevant ligand for RAGE since MG-H1 would continuously ensure full occupancy of binding sites on RAGE. More research is therefore needed on the mechanisms behind the impact of the interactions between RAGE and MGO-derived AGEs in disease.

Asides from proteins, MGO can also react irreversibly with the nucleotide deoxyguanosine to form DNA adducts, mainly N(2)-carboxyethyl-2'-deoxyguanosine (CEdG) and 3-(2-deoxyribosyl)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b]purin-9(8)one (MGdG)[45]. CEdG is thought to be the major MGO-modified nucleotide in physiological systems, as it’s relatively more

abundant *in vivo* and is more stable than MGdG[73,74]. However, the potential biological effect of MGO-derived DNA adducts remains poorly understood.

Methylglyoxal in immune cells and disease

Emerging evidence indicates that the accumulation of MGO is associated with multiple metabolic diseases, due to harmful effects of MGO. However, recent data also indicate that MGO may have beneficial effects, including a protective role against infections. Here we summarize the direct effects of MGO during infections, and its effects on different immune cells.

Methylglyoxal and infection

Methylglyoxal formation during immune cell activation

During infection, the stimulation by pathogens leads to the activation of immune cells and induces metabolic reprogramming, including a shift from OXPHOS to glycolysis[5,75-77]. In macrophages, enhanced glycolysis increases the pentose phosphate pathway (PPP), which generates nicotinamide adenine dinucleotide phosphate (NADPH) for the production of reactive oxygen species (ROS) and nitric oxide (NO). Moreover, many of the enzymes involved in glycolysis were shown to be directly involved in the activation of inflammatory pathways[78]. In addition, combined transcriptomic and metabolomics profiling identified the Krebs cycle to be broken in two places, leading to the accumulation of intermediate metabolites that contribute to inflammation and host defense[79]. One break is at the level of isocitrate dehydrogenase 1 (IDH1), an enzyme that converts citrate to alpha-ketoglutarate. The down-regulation of IDH1 leads to the accumulation of citrate, which is then rerouted to generate itaconate. Itaconate has antimicrobial properties, but also has been identified as an immunomodulatory compound as it exerts anti-inflammatory effects[80,81]. Additionally, the excess citrate is used to synthesize fatty acids, required for membrane synthesis and prostaglandin synthesis. Moreover, citrate can assist in NO production by suppling acetyl-CoA[82]. Both Itaconate and NO can inhibit succinate dehydrogenase (SDH), leading to a second break in TCA and consequent succinate accumulation. Succinate activates hypoxia-inducible factor 1α (HIF1α), which, in concert with other inflammatory stimuli like LPS, promotes inflammatory signaling, culminating in enhanced formation of inflammatory cytokines such as IL-1β[78,83]. In addition to this mechanism,

increased fatty acid oxidation also contributes to elevated secretion of IL-1β via activation NOD-like receptor *pyrin domain-containing* 3 (NLRP3) inflammasome[84,85]. Moreover, the second break of TCA cycle leads to conversion of citrate into malate via an enhanced arginosuccinate shunt, which in turn contributes to NO production[79]. Thus, these shifts from OXPHOS to glycolysis regulate immune cell function in responding to infections (Figure 2.2). The increase of inflammatory cytokine IL-1β can also contribute to the pathogenesis of insulin resistance[86] and, subsequently, of hyperglycemia[87], providing increased substrate for glycolysis in immune cells and creating a vicious cycle in obesity and type 2 diabetes (Figure 2.2).

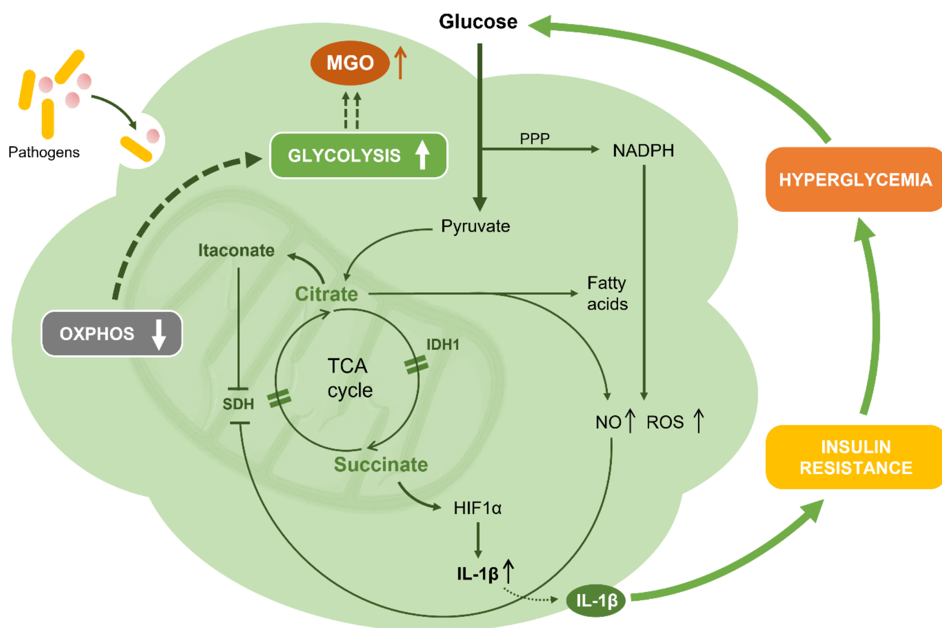

Figure 2.2 Metabolic shifts in immune cells (macrophages) during infection. OXPHOS, oxidative phosphorylation; PPP, pentose phosphate pathway; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; ROS, reactive oxygen species; TCA cycle, tricarboxylic acid cycle; IDH1, isocitrate dehydrogenase 1; SDH, succinate dehydrogenase; HIF1 α, hypoxia-inducible factor 1α; IL-1β, interleukin 1β.

In addition to the accumulation of these metabolites during infection-induced glycolysis, increasing levels of MGO will accumulate. Indeed, MGO are found to be increased during infection[88,89]. Rachman et al. demonstrated that MGO production was increased in murine alveolar macrophages after being infected

by mycobacteria *in vitro*[88]. Brenner et al. reported higher MGO levels in plasma from septic patients compared to healthy individuals and postoperative controls[89] and the potential source of MGO formation may be due to sepsis-associated hyperglycemia[90]. However, there is currently no direct evidence showing that immune cells are the source of elevated MGO during infections. An alternative source of MGO during infection could be bacteria, as they also have the ability to produce MGO[43,91]. However, since plasma MGO levels are also elevated in patients with severe trauma or following liver transplantation, conditions linked with sterile inflammation[92,93], MGO is more likely to be produced endogenously in immune cells upon the stimulation of infection[75].

In the wound healing phase of the chronic infection, anti-inflammatory macrophages are activated. Different from inflammatory macrophages, mitochondrial OXPHOS and glycolysis are both enhanced in anti-inflammatory macrophages, where is also accompanied by a complete TCA cycle. OXPHOS can be supported by glucose, possibly via fueling fatty acid synthesis to enhance fatty acid oxidation[78]. Details of metabolic reprogramming in anti-inflammatory macrophages have been reviewed in detail by Van den Bossche et al.[78]. However, the association between MGO formation and anti-inflammatory macrophages remains unclear.

Collectively, during infection, the formation of MGO is an evolutionary conserved mechanism to assist host defense. Moreover, elevated levels of MGO in activated immune cells may further exacerbate the vicious cycle of insulin resistance, hyperglycemia and enhanced glycolysis, and it cannot be ruled out that MGO might also cause type 2 diabetes as it can lead to higher fatty acid synthase activity, obesity, insulin resistance and hyperglycemia[94]. Below, we summarize the potential roles of MGO as an antimicrobial or a modulator of immune cell activity.

Direct bactericidal effects and anti-viral effects of Methylglyoxal

Several studies demonstrated that MGO has antibacterial and antiviral properties *in vitro*. Manuka honey was reported to have a significant non-peroxide antibacterial activity[95] and Mavric et al. found that Manuka honey inhibited the growth of *E.coli* and *Staphylococcus aureus* (*S.aureus*) due to the high concentrations of MGO. The minimal inhibitory concentrations of MGO ranged between 1.1-1.8 mM[96] (Table 2.1). Recently, it has been demonstrated that the antibacterial activity of Manuka honey was due to MGO-induced

inhibition of urease in bacteria. The enzyme urease catalyzes the hydrolysis of urea into carbon dioxide and ammonia, which is needed for bacterial survival in an acidic environment[97]. This inhibitory effect of urease has not been observed with other types of honey, containing less MGO[97] (Table 2.1). Consistent with these results, MGO at 0.1-1mM inhibited *E.coli* growth, an affect which lasted up to 5 h, without affecting viability after 24 hours[98] (Table 2.1). MGO also induced Ca^{2+} fluxes in *E.coli* by opening Ca^{2+} channels. This Ca^{2+} flux did not affect cell growth, but may negatively impact bacterial physiology[98,99].

Next to the effects of extracellular administration of MGO, a recent study suggested that MGO formation in neutrophils contributes to bacterial killing in Group A *Streptococcus* (GAS) infections[100] (Table 2.1). This study elegantly showed that GLO1-deficient GAS strains were unable to detoxify MGO and were more sensitive to neutrophil killing than wild type strains. Interestingly, both reducing glucose levels in the medium or blocking neutrophil myeloperoxidase (MPO) abolished the difference in survival between GLO1-deficient and wild type GAS strains[100], suggesting that MGO formation from neutrophils depends on both glucose and MPO. In this study, high concentrations of MGO (6-10mM) on GAS, were also able to kill these bacteria directly[100]. However, it remains unclear whether intracellular levels of MGO in neutrophils reaches these high concentrations. Therefore, the actual contribution of intracellularly formed MGO on bacterial killing under physiological conditions remains debated, and further studies are needed to answer this question. In addition to antibacterial effects, other studies have also reported antiviral properties of MGO, including inhibiting growth of influenza virus, inactivating viruses of foot-and-mouth disease and Newcastle disease[101-103] (Table 2.1).

Table 2.1 Direct effects of MGO during infections.

Author	Year	Topic	Outcome	Ref.
Mavric et al. Rückriemen et al.	2008 2017	Antibacterial activity of Manuka honey	Manuka honey inhibits bacteria growth by MGO	96,97
Campbell et al.	2007	Antibacterial effects of MGO	0.1-1mM MGO induces opening of Ca^{2+} channels and inhibits growth in *E.coli* without killing	98
Zhang et al.	2016	Direct bactericidal effects of MGO	Neutrophil-derived MGO contributes to bacterial killing	100
De Bock et al. Ghizatullina Tiffany et al.	1957 1976 1957	Antiviral activity of MGO	MGO inhibits the growth of influenza virus; MGO inactivates foot-and-mouth disease and Newcastle disease viruses	101-103

Methylglyoxal effects on immune cells

In addition to the direct effects of MGO on microbes, MGO may also affect immune cell recruitment. Su et al. demonstrated that exogenously administrated MGO induces endothelial activation leading to enhanced leukocyte recruitment in mice[104-106]. Incubating primary human brain microvascular endothelial cells with MGO (200 μM) led to a significant increase in monocyte adhesion, and an elevated of monocyte transmigration[107]. Thus, excessive MGO may affect the complex leukocyte-endothelial interactions by activating endothelial cells, which may contribute to immune cell recruitment and pathogen removal during infection. In the next section, we will discuss the effects of MGO on the function of different immune cells.

Neutrophils

Neutrophils are the most abundant white blood cells in humans, composing 50%~70% of circulating leukocytes[108], and are the first responders against pathogens[109]. *Ex vivo* incubation of primary neutrophils with MGO (5~30 μM) increased the release of cytokines such as TNF, IL-8, and IL-6 [110]. Neutrophils from patients with diabetes produced higher levels of cytokines compared to neutrophils from healthy individuals. Interestingly, MGO treatment of neutrophils from these patients with diabetes failed to enhance cytokine release *ex vivo,* while it did induce neutrophil apoptosis[110]. Another study found that upon co-treatment of MGO (30 μM) and high glucose (20 mM), neutrophils displayed decreased phagocytic capacity and glucose-6-phosephate dehydrogenase (G6PDH) activity, which is essential for NAPDH production[111]. This co-treatment also increased myeloperoxidase (MPO) activity and concomitant hypochlorous acid production, as well as IL-6 production[111]. In addition, cells treated with a higher concentration of MGO (1 mM) displayed enhanced ROS generation and exocytosis by increased expression of CD35 and CD66b through the activation of mitogen-activated protein kinase (MAPK) p38[112]. Together, these studies suggest that chronic exposure to MGO leads to an activated neutrophil phenotype[110,113], but at the same time makes the neutrophils resistant to further MGO-mediated activation.

Monocytes and macrophages

Monocytes constitute 5~10% of circulating leukocytes in humans. Under inflammatory conditions, monocytes are recruited to the site of injury or infection where they differentiate into macrophages[114]. *Ex vivo* MGO treatment (100 μM)

induced random migration of monocytes while the chemotactic response of monocytes to placental growth factor 1 was inhibited[115]. The mechanisms behind these effects of MGO included MGO-induced expression of SH2 domain-containing tyrosine phosphatase 2 (SHP-2) phosphate, leading to the enhanced activity of SHP-2, a regulator of growth factors and cytokine signaling[116]. Interestingly, enhanced activity of SHP-2 was also observed in monocytes from diabetic patients[115]. In addition, incubation with MGO (100 μM) induced oxidative stress in monocytic U937 cells, and 200 μM MGO induced apoptosis in these cells[117]. In the human monocytic cell line THP-1, MGO promoted apoptosis at high concentrations (1.5 mM)[118]. Moreover, 1 mM MGO led to AGE formation and inflammatory gene expression. Interestingly, MGO inhibited phagocytic function in these cells. These effects were not observed by MGO-derived AGEs[118], indicating that the inflammatory effects are linked to direct MGO action in or on the cells. However, other studies have shown inflammatory effects of MGO-modified proteins on monocytes and macrophages[65,66,119-123]. MGO-derived AGEs in bovine serum albumin induced apoptosis, CD11b expression and RAGE-dependent CD142 expression in primary human monocytes[119]. Moreover, several reports showed increased inflammatory cytokine production[65,66,120,121] and increased RAGE expression and ROS production[123] in primary monocytes and THP-1 cells by MGO-derived AGEs. In a murine alveolar macrophage cell line (MH-S cells), MGO treatment (0.8 mM) induced apoptosis, possibly by activation of c-Jun N-terminal kinase (JNK). Moreover, TNF was induced at gene and protein level, as well as the chemokine CXCL10[88]. These results are in line with pro-inflammatory effects of MGO-modified BSA on RAW 264.7 macrophages, showing enhanced ERK, p38 and NF-kB activation and TNF and ROS production[122] (Table 2.2).

Using murine models, MGO treatment increased phagocytic activity of peritoneal macrophages in tumor-bearing mice[124-126], which was linked to the anti-tumor property of MGO. In addition, MGO treatment increased peritoneal macrophage recruitment, superoxide and nitrite production, and inflammatory cytokine expression[125,126]. Moreover, iNOS and ROS production were elevated, in line with a p38 MAPK-NF-κβ and with ERK and JNK activation, respectively[125].

It is hard to disentangle the potential direct effects of MGO or MGO-derived AGEs on monocytes and macrophages. An additional layer of complexity is the rapid metabolization of MGO, which was recently illustrated in the work of Tsokanos et al.[127]. Here, they performed acute exposure (15 min) of primary

murine bone marrow-derived macrophages (BMDMs) to MGO (100µM). This induced rapid activation of the p38-MAPK and AKT/ERK pathways. Interestingly, after 60 min of MGO treatment, phosphorylation of proteins p38, ERK and AKT returned to basal levels, suggesting fast degradation of MGO, which was confirmed by measuring MGO levels directly. In addition, MGO increased glycolytic rate in these BMDMs in a dose-dependent manner. Given the fast detoxification of MGO, the authors aimed to mimic chronic MGO exposure by administrating MGO repeatedly, resulting in a distinct macrophage phenotype hallmarked by enhanced Abca1, Cd36, Pparg, IL-6 and IL-1β expression, resembling so-called metabolically activated macrophages that are associated with metabolic tissues such as adipose tissue. Interestingly, both short and long term MGO treatment failed to induce TNF expression[127].

Collectively, these data support a modulatory role of MGO in macrophage activation and potentially polarization. However, more research is needed to investigate the exact effects of different doses and incubation times of MGO on macrophage responses and phenotype.

Table 2.2 Effects of MGO and MGO-derived AGEs on immune cells.

Author	Year	Cell type	Outcome	Ref.
Wang et al.	2007	Neutrophils	Neutrophils (from controls and T2D) incubation with MGO: ↑ TNF-α, IL-8, IL-6 (only in health controls); Apoptosis	110
Guerra et al.	2012	Neutrophils	Neutrophils incubation with MGO+glucose: ↑ IL-6; hypochlorous acid production; MPO activity; ↓ Phagocytosis; G6PDH	111
Ward et al.	2004	Neutrophils	MGO incubation: ↑ P38 MAPK; ROS; CD35 and CD66b	112
Dorenkamp et al.	2018	Monocytes	MGO incubation: ↑ Random migration; SHP-2 activity; ↓ Chemotactic response to PIGF-1	115
Rom et al.	2020	Monocytes	BMVEC incubation with MGO ↑ monocyte adhesion and migration	107
Okado et al.	1996	Monocytic U937 cells	MGO incubation: ↑ Apoptosis; oxidant stress	117
Bezold et al.	2019	THP-1 macrophages	MGO incubation: ↑ Surface glycation; AGE; IL-1β (M1), IL-8 (M1/M2), TNF-α (M1/M2), IL-10 (M2); apoptosis (only >1.5mM MGO); ↓ Phagocytic efficiency	118

Table 2.2 (continued)

Author	Year	Cell type	Outcome	Ref.
Chakrabarti et al.	2014	Peritoneal macrophages	Intravenous MGO: ↑ Peritoneal macrophage count; Phagocytosis; superoxide level; nitrite; iNOS; cytokine production	126
Rachman et al.	2006	MH-S macrophages	MGO incubation: ↑ Apoptosis; JNK activation; TNF-α; CXCL10	88
Tsokanos et al.	2021	Bone marrow-derived macrophages (BMDM)	MGO incubation: ↑ p-p38, p-ERK, p-AKT (acute treatment); glycolysis (acute treatment); Abca1, Cd36, Pparg, IL-6, IL-1β (prolonged treatment)	127
Bhattayharyya et al. Pal et al.	2008 2009	Peritoneal macrophages Lymphocytes T cells	Oral MGO treatment: ↑ peritoneal macrophages; Phagocytic capacity; ROIs; RNIs; NADPH oxidase; iNOS; inflammatory signaling and cytokines ↑ $CD4^+$ and $CD8^+$ T cell activation	124 125
Du et al. Takagi et al.	2000 2004	Lymphocytes Jurkat T cells	MGO incubation: ↑ Apoptosis; ↓ Mitochondrial membrane potential	129 130
Baumann et al.	2020	Lymphocytes T cells	MGO producing MDSCs with CD8+ T cells co-culture: ↓ activation	133
Price et al.	2010	Lymphocytes T cells	MGO incubation: ↓ $CD8^+$ T cell activation; IFN-γ	128
Rosenstock et al.	2019	Lymphocytes NK-92 cells	MGO incubation: ↑ Glycation; ↓ Cytotoxic function; Metabolic activity; Aggregate formation	136
Gawlowski et al.	2007	Neutrophils Monocytes	Whole blood incubation with MGO / AGE-BSA: ↑ Platelet P-selection (only with MGO); Platelet-neutrophil aggregates; Apoptosis; Mac-1 (CD11b) PBMCs incubation with MGO / AGE-BSA: ↑ CD142 (tissue factor);	119
Westwood et al. Abordo et al. Webster et al. Abordo et al.	1996 1997 1997 1996	Monocytes; Monocytic THP-1 cells	Human monocytes or THP-1 cells incubation with MGO-HSA: ↑ IL-1β; TNF-α; M-CSF	66 65 121 120
Rondeau et al.	2008	Monocytic THP-1 cells	Incubation with MGO-BSA/-HSA: ↑ RAGE; ROS	123
Fan et al.	2003	RAW 264.7 macrophages	Incubation with MGO-BSA: ↑ TNF-α; ROS; ERK1/ERK2 and p38 MAPKs; NF-κB	122

Lymphocytes

Lymphocytes make up 18%~42% of circulating leukocytes and consist of B- and T-cells, the major components of adaptive immune system, and of NK cells, which are innate immune cells. Oral MGO administration to tumor bearing mice increased the number of CD8 and CD4 positive T cells in spleen[125], and elevated the cytotoxicity of splenic lymphocytes[124]. In contrast, *ex vivo* experiments showed that MGO (500 μM) inhibited the proliferation of CD8+ T cells by damage to DCs[128]. In line with these latter data, IFN-γ production in CD4+ and CD8+ T cells was found to be reduced after MGO incubation with peripheral blood mononuclear cells (PBMCs)[128]. Additionally, *ex vivo* incubation with MGO (250 μM) induced apoptosis in Jurkat leukemia T cells by activation of JNK, inducing mitochondrial membrane potential loss, caspase-3 activation and PARP cleavage[129]. A follow-up study showed that MGO-dependent cell death was prevented by phorbol 12-myristate 13-acetate - induced ERK activation that blocked JNK-induced cytochrome c leakage from mitochondria[130].

The reasons for these discrepancies between the *in vivo* and *in vitro* experiments remain unclear. Although it was recently shown that dietary intake of MGO is associated with plasma MGO level, the portion of orally administrated of MGO to taken up in the intestine is not known[39]. Moreover, MGO may react with dietary proteins to form MGO-derived AGEs before absorption. It has been shown that increased dietary AGEs affect intestinal permeability and hence leakage of pro-inflammatory LPS as well as induce changes in intestinal flora, leading to increased inflammation[131]. In line, we recently showed that an increased load in dietary AGEs induces reversible changes to microbiome, accompanied by increased inflammation, most notably related to decreased circulating IL-10 levels[132]. Also in humans, evidence was found that a bolus of oral MGO is rapidly degraded during the digestion process and exerts little effect on *in vivo* MGO levels[40]. Thus, it is possible that the effects of *in vivo* oral MGO administration observed are secondary to other mechanisms. It is also possible that effects of MGO are different depending on the dosage, and that the amount of MGO reaching the cells *in vivo* is relatively low compared to levels used in *in vitro* experiments. In support of this, opposed to the administration of exogenous MGO, Baumann et al. proved that endogenously formed MGO in myeloid-derived suppressor cells (MDSCs) is transferred directly to T cells in a cell contact-dependent fashion[133]. This direct transfer of MGO inhibited T-cell effector functions in inflamed or cancerous tissues, suppressed T cell proliferation and inhibited the expression of the cytokines TNF and IFN- γ. The

mechanism behind this effect was the rapidly reaction of MGO with L-arginine in T cells leading to depletion of L-arginine[133], which is required for T cell activation[134,135]. Interestingly, MDSC-delivered MGO inhibited glucose uptake in effector CD8+ T cells[133].

Finally, *ex vivo* incubation of MGO with the human NK cell line NK-92 led to increased glycation of cellular proteins in a concentration-dependent manner (0-2 mM). High doses of MGO (1mM, 2mM) reduced metabolic activity by 40%, as well as their cytotoxic function[136].

Conclusions and perspective

The production of MGO during glycolysis has been mainly viewed as a side product occurring in chronic metabolic disease and involved in the development of diabetes and its vascular complications. However, emerging insights in immunometabolism and the proven importance of increased glycolysis during inflammatory activation, suggests that the production of MGO and MGO-derived AGEs could represent an evolutionary conserved mechanism. Indeed, MGO has direct antimicrobial properties, and many studies suggest an immunomodulatory role in several immune cells. However, many questions remain unaddressed. It remains to be determined whether intracellular levels of MGO found *in vivo* are high enough to exert the putative antimicrobial functions. In several studies, the effects of MGO on immune cells showed an increase of inflammation, apoptosis, and suppressed phagocytosis in neutrophils, monocytes and macrophages, while in lymphocytes, MGO inhibited their activity. However, it remains challenging how to connect these potential effects of MGO to diseases.

In addition, although most MGO is derived from glucose and glycolysis, enhanced lipid metabolism and protein metabolism can contribute to its formation[32,34]. Although changes in lipid and protein metabolism are important during immune cell activation, very little is known about the interaction between lipid and protein metabolism and MGO formation during immune cell activation. In turn, MGO itself may also affect lipid metabolism. A study in a *Drosophila* model showed that elevated levels of MGO is not only a consequence of hyperglycemia, but can also induce elevated fatty acid synthase activity, and contribute to the progress of obese, insulin resistance and hyperglycemia[94]. Lipid peroxidation is also a source of MGO, and fatty acid synthesis plays

essential roles in immune cell metabolic shifts and participates in inflammation[137]. Given the known impact of chronic inflammation in the development of insulin resistance this raises the question whether MGO formation in activated immune cells may contribute to the development in type 2 diabetes, which will be needed to study in future research.

Moreover, current recognition regarding the effects of MGO on immune cell recruitment and function remain debatable due to several reasons. It is of importance to consider differences between endogenously produced MGO and exogenously administrated MGO. Moreover, many studies have used different concentrations of MGO and different incubation times in their experiments, which may exert different effects. MGO is rapidly degraded intracellularly and can rapidly interact with proteins in the plasma and in culture medium. Despite the enormous progress made in recent years further research it should be emphasized that for a proper design and correct interpretation of data, experimental work needs to be done with highly purified batches of MGO, with physiological concentrations of MGO and with proteins minimally modified by MGO. A point of concern is that so far, all the experiments with MGO use high concentrations of a commercially available batch of MGO, which is known to be contaminated by aldehydes that potentially interfere in studies with MGO. Therefore, we cannot exclude the possibility that the effects are due to other compounds present in the MGO batch used for the experiments.

In order to determine the precise role of MGO during immune cell activation and host defense, well controlled studies using highly purified MGO with a concentration that are relevant for the *in vivo* situation are therefore warranted.

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Chapter 3

Increased methylglyoxal formation in plasma and tissues during a glucose tolerance test is derived from exogenous glucose

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Abstract

The dicarbonyl compound methylglyoxal (MGO) is a major precursor in the formation of advanced glycation endproducts (AGEs). MGO and AGEs are increased in subjects with diabetes and are associated with fatal and non-fatal cardiovascular disease. Previously we have shown that plasma MGO concentrations rapidly increase in the postprandial phase, with a higher increase in individuals with type 2 diabetes. In current study, we investigated whether postprandial MGO formation in plasma and tissues originates from exogenous glucose and whether the increased plasma MGO concentration leads to a fast formation of MGO-derived AGEs.

We performed a stable isotope labelled oral glucose tolerance test (OGTT) in 12 healthy males with universally labelled D(+)^{13}C glucose. Analysis of plasma labelled $^{13}C_3$ MGO and glucose levels at eleven time-points during the OGTT revealed that the newly formed MGO during OGTT is completely derived from exogenous glucose. Moreover, a fast formation of protein-bound MGO-derived AGEs during the OGTT was observed. In accordance, *ex vivo* incubation of MGO with plasma or albumin showed a rapid decrease of MGO and a fast increase of MGO-derived AGEs. In an intraperitoneal glucose tolerance test in C57BL/6J mice, we confirmed that the formation of postprandial MGO is derived from exogenous glucose in plasma and also showed in tissues that MGO is increased and this is also from exogenous glucose.

Collectively, increased formation of MGO during a glucose tolerance test arises from exogenous glucose both in plasma and in tissues, and this leads to a fast formation of MGO-derived AGEs.

Introduction

Methylglyoxal (MGO) is a highly reactive dicarbonyl compound and spontaneously modifies amino groups in protein. MGO is a major precursor in the formation of advanced glycation endproducts (AGEs)[1] such as Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1) and N^{ε}-(carboxyethyl)lysine (CEL)[2-4]. MGO and MGO-derived AGEs have detrimental effects on cellular function[5-7], inflammation and cell death[8-10], are increased in subjects with type 1 and 2 diabetes and are associated with fatal and non-fatal cardiovascular disease[8,11-15]. We have recently shown that plasma MGO concentrations rapidly increased both during an oral glucose tolerance test (OGTT) and a mixed meal test, with a higher increase in individuals with type 2 diabetes[16,17]. Since MGO is rapidly formed postprandially, repeated episodes of elevated MGO plasma concentration may lead to MGO stress, and may contribute to the detrimental effects of postprandial glucose spikes[18].

MGO is mainly formed from glycolysis-derived triose phosphates[19,20], but minor sources are degradation of glycated proteins, oxidation of acetone and lipid peroxidation[13]. The exact source of the postprandial MGO in plasma is unknown. Therefore, we investigated *in-vivo* whether postprandial MGO directly originates from exogenous glucose during an OGTT in healthy humans, using universally labelled D(+)^{13}C glucose, and in mice whether MGO increased in tissues after a GTT. Furthermore, we investigated *in-vitro* whether the MGO leads to a rapid formation of plasma MGO-derived AGEs.

Materials and methods

Human study

A total of 12 healthy males, with an average age of 25 years (range 21-30 years) and average BMI of 22.5 kg/m^2 (range 19.2-24.7 kg/m^2) were recruited. No medication use was reported by the subjects. Approval was obtained from the Medical Ethics Committee Brabant (Tilburg, The Netherlands) on 1 December 2015. Each subject provided written informed consent for the study. This trial was registered at controlled-trials.com as ISRCTN42106325.

Experimental design

After an overnight fasting period, study participants underwent a stable isotope labelled OGTT with 50 g glucose of which 2% was universally labelled D(+)^{13}C glucose, enabling us to track the occurrence of labelled $^{13}C_3$ MGO, which we refer to as formation of MGO, in the obtained samples. Blood samples were collected at eleven time-points after the intake of glucose: every 15 min for first two hours and every half an hour for the next four hours. Concentrations of MGO and glucose in plasma during OGTT (6 h) were measured at eleven time-points with ultra-performance liquid chromatography-tandem mass spectrometry.

Measurements of MGO, free and protein-bound MGO-derived AGEs and plasma glucose

MGO and MGO-derived AGEs in plasma were measured with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS, Waters, Milford, USA), as described in detail previously[21,22]. Plasma glucose concentrations were measured on a Roche/Hitachi Modular automatic analyzer (Roche Diagnostics, Hitachi) by using a glucose hexokinase method. $^{13}C_3$ MGO and $^{13}C_6$ glucose data were corrected for a difference in mass spectrometry response factor between $^{13}C_3$ MGO and $^{12}C_3$ MGO, and between $^{13}C_6$ glucose and $^{12}C_6$ glucose, respectively.

It was assumed that labelled and unlabelled glucose molecules showed identical behaviour. Based on the 2% dose of the total of 50 g glucose, the appearance of total $^{13}C_6$ glucose and $^{13}C_3$ MGO was corrected for dose.

Ex vivo experiments

Bovine serum albumin (50 g/L in PBS) and human plasma from healthy donors was incubated at 37°C for 0, 1, 2, 3, 4, 5, 6, 8 and 24 hours with 0, 1, 10, and 100 µmol/L MGO. MGO was produced as previously described[23]. Every sample was snap frozen in liquid nitrogen and stored at -80°C until analysis. Protein-bound MG-H1 and CEL levels and MGO concentrations were measured with UPLC-MS/MS (Waters, Milford, USA)[21,22].

Mouse experiments

4-week-old male C57BL/6J mice were purchased from Charles River Europe and were maintained in the animal facility until the age of 10-12 weeks, with 4 mice per cage. Mice were fasted overnight for 16 h and received an

intraperitoneal glucose tolerance test (IPGTT) with a solution of universally labelled D(+) ^{13}C glucose (Sigma-Aldrich), 2g/kg body weight dissolved in saline. These mice were euthanized by means of CO_2/O_2 mixture at either 30, 60, or 120 min after the glucose injection, respectively. The control group of mice received no treatment and were euthanized directly, at time 0 min of the IPGTT. Blood samples were collected from the heart in EDTA tubes and stored immediately on ice. Plasma samples were obtained after spinning the blood at 2000g, 4°C for 5 min, and were snap frozen and stored at -80°C until analysis. Pancreas, spleen, liver, kidney, visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), and skeletal muscle were collected, snap frozen and stored at -80°C. Glucose levels were measured in whole blood using a glucometer (Contour, Bayer, Leverkusen, Germany).

The experimental work protocol was approved by the local Animal Experiments Committee of Maastricht University, project license number AVD1070020187086. Experiments were performed at the Animal Laboratory of Internal Medicine, Maastricht University, by licensed people according to institutional guidelines.

Tissue preparations for measurements

Pancreas, spleen, liver, kidney, and skeletal muscle were homogenized by crushing in liquid nitrogen and dissolved in lysate buffer (0.1M sodium phosphate buffer supplemented with 0.02% Triton-x (Sigma-Aldrich) and protease inhibitor (Roche)). VAT and SAT were homogenized with a Mini-bead beater homogenizer (Biospec), in the same lysate buffer as used for the other tissues. Concentrations of $^{13}C_3$ MGO and $^{12}C_3$ MGO were measured in homogenates of all tissue and plasma samples using UPLC-MS/MS, as described previously[21].

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis were performed using GraphPad Prism 8.0.2. Two-way ANOVA with Bonferroni's multiple comparisons test was used to compare groups over time. One-way ANOVA with Tukey's multiple comparisons test was used to compare MGO and glucose levels between different time points. All analysis were considered statistically significant with p values <0.05.

Results

Postprandial MGO formation in plasma during an OGTT is derived from exogenous glucose in humans

To investigate whether postprandial MGO formation directly originates from exogenous glucose, we performed an OGTT with 50 g glucose of which 2% was universally labelled D(+)^{13}C glucose. Plasma $^{12}C_6$ glucose levels increased rapidly after the glucose load with a calculated peak at 41 min, after which levels started to decline. The plasma $^{13}C_6$ glucose concentration showed a similar peak value as for $^{12}C_6$ glucose. However, the decline of $^{13}C_6$ glucose levels after the peak was significantly slower than for $^{12}C_6$ glucose (Table 3.1, Figure 3.1A). After 180 min post-load, plasma $^{12}C_6$ glucose, but not $^{13}C_6$ glucose, slightly increased. (Figure 3.1A). The curves of plasma $^{12}C_3$ MGO and $^{13}C_3$ MGO followed the same pattern as observed for $^{12}C_6$ glucose and $^{13}C_6$ glucose, respectively. Plasma $^{12}C_3$ MGO and $^{13}C_3$ MGO showed a rapid increase with a calculated Cmax at 49 min and 56 min, respectively (Table 3.1, Figure 3.1B). After 180 min, $^{12}C_3$ MGO slightly increased (Figure 3.1B). Collectively, these data show that the rapid increase of MGO formation is completely derived from exogenous glucose during the 6-hour OGTT.

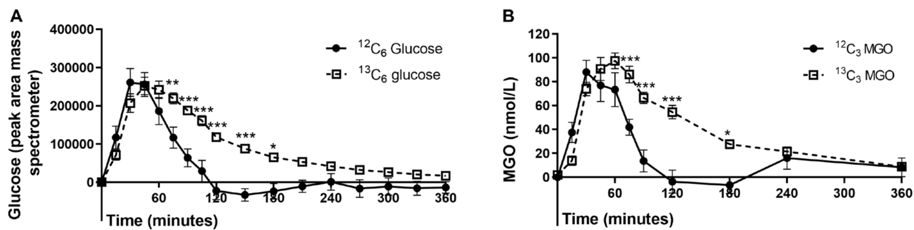

Figure 3.1 Plasma glucose and methylglyoxal concentrations during a stable isotope labelled OGTT with universally labelled D(+)$^{13}C_6$ glucose in humans. (**A**) Plasma glucose levels, and (**B**) Plasma MGO concentrations were analysed by UPLC-MS/MS. $^{13}C_6$ glucose and $^{13}C_3$ MGO data were corrected for dose (*50) and for a difference in mass spectrometry response factor between $^{13}C_6$ glucose and $^{12}C_6$ glucose, $^{13}C_3$ MGO and $^{12}C_3$ MGO, respectively. $^{12}C_6$ Glucose and $^{12}C_3$ MGO levels were corrected for baseline. Data are shown as mean ± SEM, n=12. Comparisons of $^{13}C_6$ glucose and $^{12}C_6$ glucose, $^{13}C_3$ MGO and $^{12}C_3$ MGO over time were tested using two-way ANOVA with Tukey's multiple comparison. * represents comparisons with glucose or MGO at the same time point. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. MGO, methylglyoxal.

Table 3.1 Cmax and Tmax of glucose and MGO.

Parameter	Cmax* (mean ± SEM, unit)	Tmax (mean ± SEM, unit)
$^{12}C_6$ glucose	328114 ± 40611 (peak area MS)	41 ± 4.1 (min)
$^{13}C_6$ glucose	298345 ± 17997 (peak area MS)	50 ± 4.3 (min)
$^{12}C_3$ MGO	110.8 ± 11.8 (nmol/L)	48 ± 4.9 (min)
$^{13}C_3$ MGO	109.7 ± 6.5 (nmol/L)	56 ± 4.9 (min)

* $^{12}C_6$ glucose and $^{12}C_3$ MGO were corrected for baseline, $^{13}C_6$ glucose and $^{13}C_3$ MGO were corrected for dose (*50). MS, mass spectrometry.

Postprandial protein-bound AGEs formation in plasma increases rapidly during an OGTT in humans

Since MGO can react rapidly with proteins to form AGEs[24], we next investigated the formation of postprandial MGO-derived AGEs in plasma during the 6h OGTT. Plasma protein-bound CEL increased after a glucose load and reached a peak 60 min (+85%; $p<0.001$). Plasma protein-bound MG-H1 showed the highest levels after 120 minutes (+8%; $p=0.36$), but this was not statistically significant. For both MGO-derived AGEs, the concentrations decreased and the lowest concentrations were found at 240 min during the OGTT, after which the concentrations slightly increased again (Figure 3.2A and 3.2B). In contrast to protein-bound AGEs, the concentrations of free plasma MG-H1 and CEL continuously decreased during the entire 6h OGTT (Figure 3.2C and 3.2D).

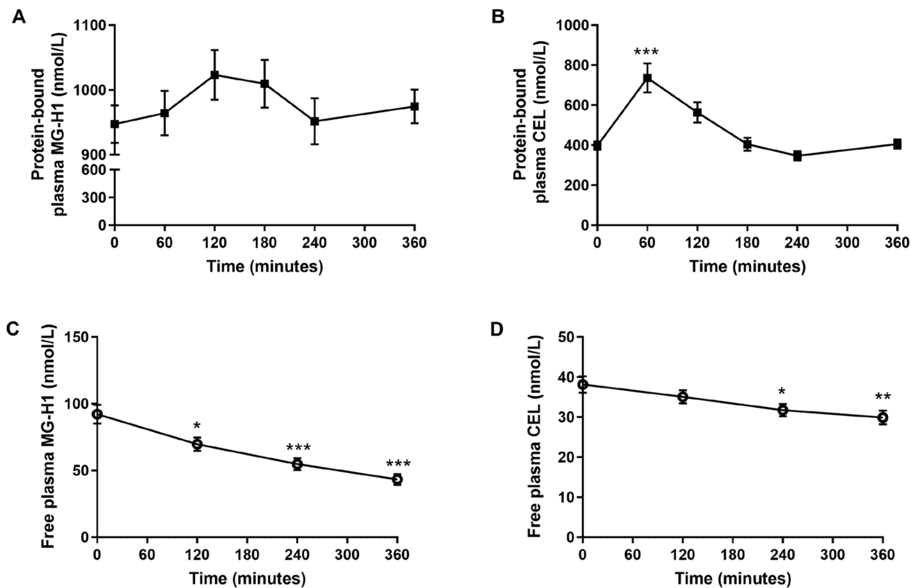

Figure 3.2 MGO-derived AGEs concentrations in plasma during an OGTT in humans. (A) Protein-bound plasma MG-H1, (B) protein-bound plasma CEL, (C) free plasma MG-H1, and (D) free plasma CEL were analysed by UPLC-MS/MS. Data are shown as mean ± SEM, n=12. Comparisons of protein-bound and free AGEs levels at different time points were tested using one-way ANOVA with Tukey's multiple comparison. * represents comparisons with time 0. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. MG-H1, Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; CEL, N^{ε}-(carboxyethyl)lysine.

MGO rapidly induces AGEs formation in plasma *ex vivo*

We next investigated whether these MGO-derived AGEs can be directly formed by MGO in plasma. The incubation of human plasma with different concentrations of MGO *ex vivo* for 24h, induced a time- and dose-dependent increase of protein-bound MG-H1 and CEL, which occurred rapidly during the first 6 hours (Figure 3.3A and 3.3B). After 6 hours, the levels of protein-bound MG-H1 and CEL continued to increase, albeit at a slower rate.

The incubation of bovine serum albumin (BSA) with MGO resulted in a similar pattern for protein-bound MG-H1 formation as in plasma, while protein-bound CEL formation was not induced by MGO (Figure 3.3C and 3.3D). The incubation of BSA with 10 µM and 100 µM MGO showed a fast decrease of MGO levels during the first 6 hours (Figure 3.3E and 3.3F), demonstrating the high reactivity of MGO with proteins.

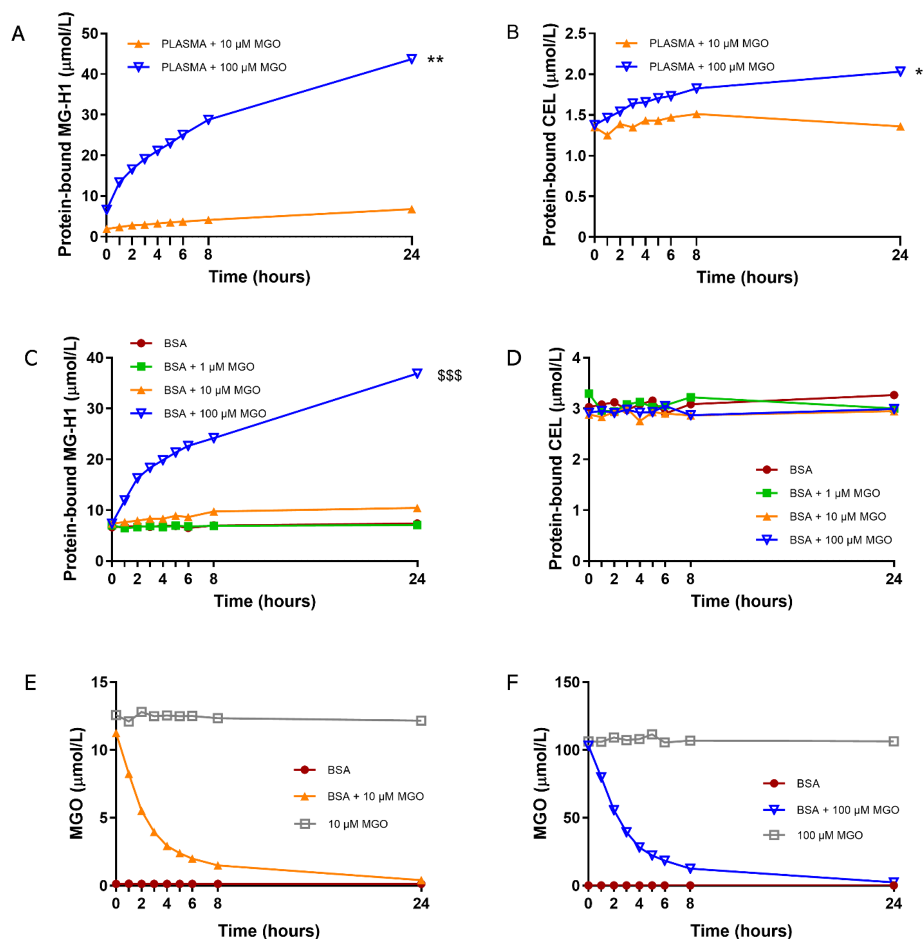

Figure 3.3 Protein-bound AGE formation in plasma and BSA after incubating with MGO *ex vivo*. The concentrations of (A) protein-bound plasma MG-H1, (B) protein-bound plasma CEL, (C) protein-bound BSA MG-H1, (D) protein-bound BSA CEL, and MGO after (E) 10 µM MGO incubation and (F) 100 µM MGO incubation were measured by UPLC-MS/MS. Comparisons of MGO effects on protein-bound MG-H1 or protein-bound CEL formation were tested using two-way ANOVA with Bonferroni's multiple comparison. * represents comparisons with 10 µM MGO, $^{\$}$ represents comparisons with BSA. * indicates p<0.05, ** indicates p<0.01, and $^{\$\$\$}$ndicates p<0.001. MGO, methylglyoxal; MG-H1, Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; CEL, N^{ε}-(carboxyethyl)lysine; BSA, bovine serum albumin.

MGO formation in tissues during an intraperitoneal glucose tolerance test is also derived from exogenous glucose in mice

Next, we studied in mice whether exogenous glucose contributes to *in vivo* MGO formation in different organs after an intraperitoneal glucose bolus of universally labelled D(+)^{13}C glucose. Blood glucose levels increased rapidly after the intraperitoneal glucose injection and reached a peak at 30 min (Figure 3.4A). Plasma $^{13}C_3$ MGO levels (Figure 3.4B) followed the same trend as glucose, which is in accordance with the findings in humans (Figure 3.1B). In pancreas, spleen, kidney, subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT), $^{13}C_3$ MGO concentrations increased after the glucose bolus, with a peak at 30 min. After this peak, $^{13}C_3$ MGO levels declined, with some residual $^{13}C_3$ MGO in the tissues at 120 min (Figure 3.4C-G). In the liver and muscle, $^{13}C_3$ MGO formation also increased during the IPGTT with a peak at 60 min (Figure 4H-I). Non-labelled MGO levels in all the tissues during IPGTT were not affected by the bolus of glucose or slightly decreased at later time points (Figure S3.1). Taken together, these data in mice showed that, similar to plasma, MGO transiently accumulates in tissues during a glucose tolerance test, and that its formation is directly derived from exogenous glucose.

Discussion

Our study demonstrates that postprandial plasma MGO formation during an OGTT originates from exogenous glucose. This formation of MGO during an OGTT is followed by increased formation of MGO-derived AGEs in plasma. *Ex vivo* experiments showed that MGO is rapidly decreased when incubated with plasma or BSA, accompanied by a fast formation of MGO-derived AGEs. In mice, we showed that not only the postprandial increase of plasma MGO is derived from exogenous glucose, but also MGO formation in pancreas, spleen, kidney, SAT, VAT, liver, and skeletal muscle.

Under physiological conditions, MGO is mainly formed during glycolysis by the non-enzymatic degradation of the glucotrioses glyceraldehyde 3-phosphate and dihydroxyacetone phosphate[13,19], but MGO can also be produced by auto-oxidation of glucose and degradation of glycated proteins[25], catabolism of threonine and acetoacetate[26,27], and lipid peroxidation[28]. In addition to these endogenous sources, MGO can be ingested from food[29]. We previously showed that MGO levels increased rapidly in plasma during an OGTT and a mixed meal

test, and that MGO levels followed the trend of glucose curves[16,17], suggesting that increased levels of postprandial glucose are the source of MGO formation in the postprandial phase. Here, we established that the formation of MGO during the OGTT is indeed completely derived from exogenous glucose.

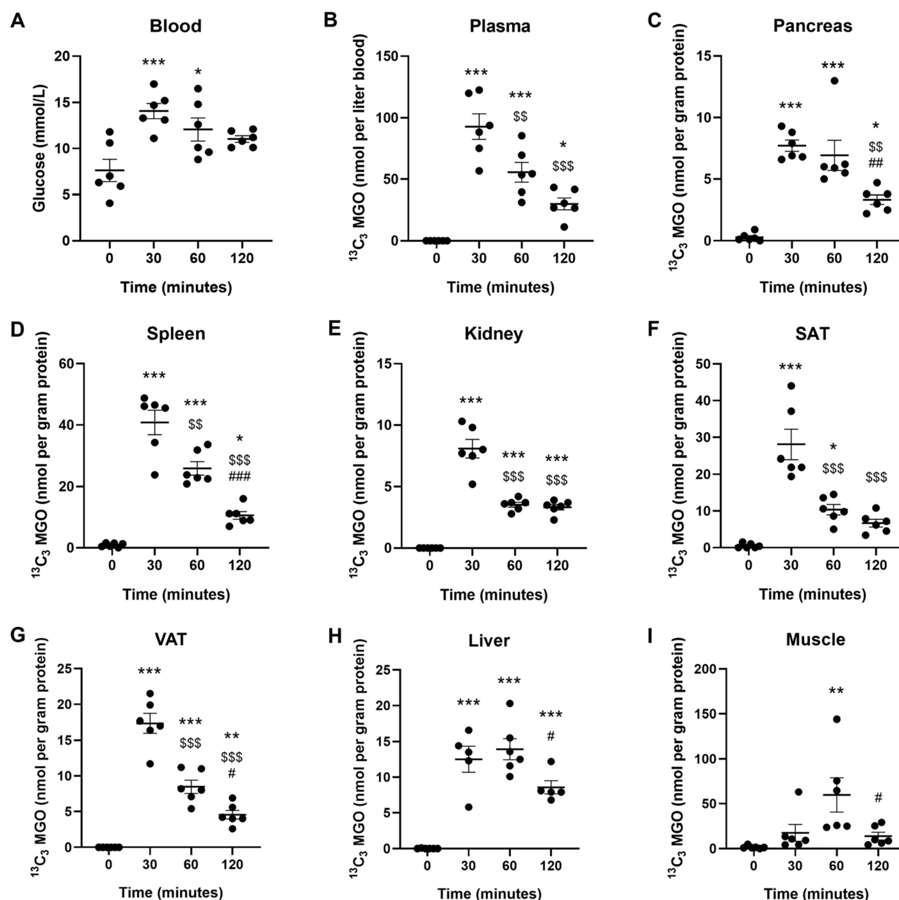

Figure 3.4 Blood glucose levels and $^{13}C_3$ MGO levels in tissues during a 2-hour IPGTT in mice. Glucose levels were measured using a glucometer in (**A**) whole blood. $^{13}C_3$ MGO concentrations were analysed by UPLC-MS/MS, in (**B**) plasma, (**C**) pancreas, (**D**) spleen, (**E**) kidney, (**F**) SAT, (**G**) VAT, (**H**) liver, and (**I**) muscle tissue from C57BL/6J male mice, 10-11 weeks old. Mice were grouped for different time points, n=6 per group. Data are shown as mean ± SEM. Comparisons of glucose levels or $^{13}C_3$ MGO levels at different time points were tested using the ordinary one-way ANOVA with Tukey's multiple comparison. * represents comparisons with time 0, $ represents comparisons with time 30 min, and # represents comparisons with 60 min. */$/# indicates $p<0.05$, **/$$/## indicates $p<0.01$, and ***/$$$/### indicates $p<0.001$. SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

The decrease of $^{13}C_6$ glucose and $^{13}C_3$ MGO levels in plasma after the peak was slower as compared to unlabelled glucose and MGO within 180 min of OGTT, which is most likely due to an overcorrection of the ^{12}C curve in the postprandial phase. The baseline level of ^{12}C glucose is approximately 5 mmol/L and reflects the balance between endogenous production and disposal in fasting state. However, the endogenous production declines rapidly in the postprandial state due to the effects of insulin, to ~35% of baseline by ~60 min[17]. By subtracting a fixed baseline level of 5 mmol/L from the total curve of $^{12}C_6$ glucose this over-corrects the ^{12}C curve, explaining a lower $^{12}C_6$ glucose compared to $^{13}C_6$ glucose curve. This effect becomes most apparent after ~30-60 min. The same effects play a role for $^{12}C_3$ MGO. The baseline $^{12}C_3$ MGO levels probably reflect the endogenous production of glucose in the liver and muscles by oxidation of glucose/glycogen, which are processes that are also reduced in the postprandial phase. In support of this concept, we observed lower levels of $^{12}C_3$ MGO in mice during the postprandial phase in several tissues including the liver and the muscle. Correction for the baseline MGO levels will again overcorrect the $^{12}C_3$ MGO curve. Although we cannot exclude the possibility that there is also a kinetic isotope effect resulting in lower rates of utilization of $^{13}C_6$ glucose for glycolysis[30], such an effect is likely too small to explain the observed difference between ^{12}C and ^{13}C patterns of glucose and MGO. In addition, it was also observed that unlabelled glucose and MGO mildly increased again after 180 min of the OGTT, which could be due to a restoration in endogenous glucose formation after being suppressed in the postprandial state.

In agreement with our current findings, we have previously shown a similar increase of the MGO levels in plasma in healthy individuals during OGTT, with a higher increase of plasma MGO in individuals with type 2 diabetes as compared with healthy subjects[17]. Given that glucose spikes have been established as a key driver in the development of diabetic vascular complications[13,18], daily spikes of MGO, as a consequence of postprandial glucose peaks, may explain the effects of glucose spikes.

MGO can react with proteins, resulting in a fast formation of AGEs[24]. Indeed, in the OGTT in humans we showed a fast increased formation of protein-bound CEL and MG-H1, although the later to a lesser extent. In the *ex vivo* experiments, we confirmed MGO-induced formation of MG-H1 and CEL in plasma was in a concentration and time-dependent fashion. Interestingly, an incubation of MGO with pure BSA produced MG-H1, but failed to induce protein-

bound CEL within 6 hours. Since we observed MGO-induced CEL formation in plasma, and since CEL was most profoundly increased during the *in vivo* OGTT in human subjects, the reaction of MGO with lysine in plasma may preferentially be on proteins other than albumin. The low appearance of MG-H1 in plasma *in vivo*, in comparison to the high increase of the formation of MG-H1 *ex vivo*, might be due to a rapid degradation of MG-H1 containing proteins *in vivo* by epithelial cells of renal tubular[31]. In contrast to protein-bound MG-H1 and CEL, free MG-H1 and CEL were decreased over time during the OGTT. We previously found a strong association between dietary AGEs and free AGEs, but not with protein-bound AGEs[32]. Therefore, this decrease of free MG-H1 and CEL during the OGTT most likely reflects the process of the clearance of free AGEs that were derived from food before fasting.

In line with the findings in humans, changes of plasma $^{13}C_3$ MGO levels followed the same pattern as of glucose during IPGTT in mice. Furthermore, an elevation of $^{13}C_3$ MGO levels but not $^{12}C_3$ MGO, after a bolus of glucose was observed in pancreas, spleen, kidney, SAT, VAT, liver, and muscle, indicating that the increase of MGO in tissues was completely derived from exogenous glucose. The changes of $^{13}C_3$ MGO in the pancreas, spleen, kidney, SAT, and VAT followed the same pattern as for plasma $^{13}C_3$ MGO. Thus, exogenous glucose rapidly results in MGO formation in various tissues. It has been previously demonstrated that glycolysis was largely increased in SAT in the postprandial phase, with only a small increase in pancreas and kidney, and no change in spleen and VAT[33]. Therefore, it might be that the elevated $^{13}C_3$ MGO concentration in spleen and VAT is due to uptake of $^{13}C_3$ MGO from plasma, rather than from glycolysis. The concentrations of $^{13}C_3$ MGO in liver and muscle also increased during the IPGTT, however, with some delay compared to the other tissues. These tissues are very insulin dependent for the uptake of glucose and, therefore, this delay in the formation of $^{13}C_3$ MGO in liver and muscle may reflect the kinetics of insulin release by the pancreas[34]. Another explanation for the relatively later increase of $^{13}C_3$ MGO in the liver and skeletal muscle may be that the intake of glucose is not directly used for glycolysis but first absorbed and used into the glycogen pool, that was likely emptied due to the overnight fasting, before being used for glycolysis[33]. The fact that hepatic $^{12}C_3$ MGO levels declined during IPGTT may be explained by a lower use of the glycogen contribution to glycolysis in the liver.

A limitation of the current study is that only male participants were enrolled in the human study and because of gender differences in response to an OGTT[35], these data may not be completely extrapolated to women.

Overall, we found that postprandial MGO formation during a glucose tolerance test is directly derived from exogenous glucose both in plasma and in tissues, followed by a fast formation of MGO-derived AGEs. MGO stress in the postprandial phase may contribute to the detrimental effects and long-term complications due to postprandial glucose spikes. Reducing MGO stress in the postprandial phase may be a way to reduce the burden of cardiovascular disease in diabetes.

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Supplementary data

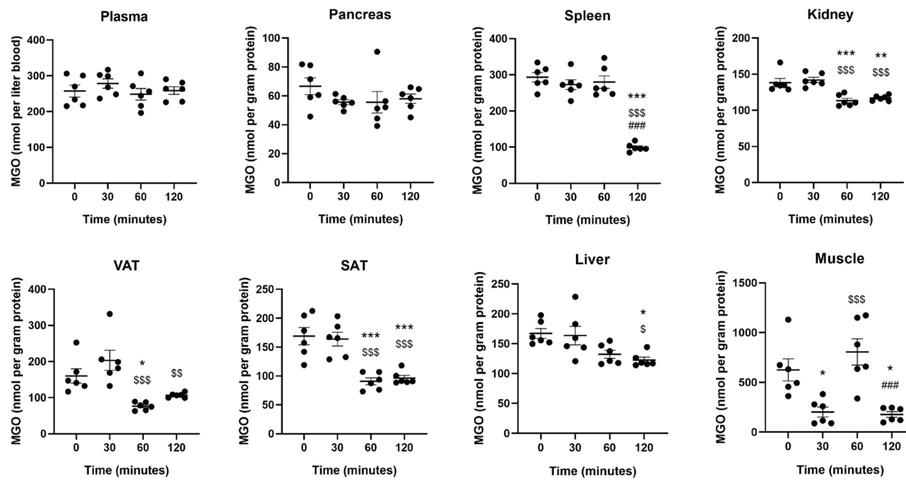

Figure S3.1 MGO levels in tissues during a 2-hour IPGTT in mice. MGO concentrations were analysed by UPLC-MS/MS, in plasma, pancreas, spleen, kidney, SAT, VAT, liver, and muscle tissues from C57BL/6J male mice, 10-11 weeks old. Mice were grouped for different time points, n=6 per group. Data are shown as mean ± SEM. Comparisons of MGO levels at different time points were tested using the ordinary one-way ANOVA with Tukey's multiple comparison. * represents comparisons with time 0, $ represents comparisons with time 30 min, and # represents comparisons with 60 min. */$/# indicates *p*<0.05, **/$$/## indicates *p*<0.01, and ***/$$$/### indicates *p*<0.001.

Chapter 4

The formation of exogenous glucose-derived methylglyoxal in circulating immune cells and in tissues is increased in obesity during a glucose tolerance test

EMBARGOED

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Ready for submission

Chapter 5

Assessment of the contribution of glycolysis to the exogenous glucose-derived methylglyoxal formation in mice

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In preparation

Chapter 6

Methylglyoxal has no direct effect on immune cell counts or activation in blood and liver, but is involved in trained immunity

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In preparation

Chapter 7

Fasting plasma methylglyoxal concentrations are associated with higher numbers of circulating intermediate and non-classical monocytes but with lower activation of intermediate monocytes: The Maastricht Study

EMBARGOED

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Chapter 8

Summary and general discussion

Summary

People with type 2 diabetes (T2D) are at high risk of developing cardiovascular diseases and low-grade inflammation is thought to be an important contributor. Inflammatory response involves innate immune cell recruitment and activation. Alterations in immunometabolism and induction of trained immunity in innate immune cells are linked to hyperglycaemia and dysregulation of these cells are implicated in diabetes-related pathological conditions. In addition, elevated methylglyoxal (MGO) levels are observed under hyperglycaemia conditions. **Chapter 1** introduced the alterations of immune cells and MGO in low-grade inflammation, as well as their potential role in T2D and its complications. Abnormal metabolism of MGO is linked with inflammation and epigenetic changes, which may contribute to cardiovascular disease. This thesis mainly aims to investigate the interactions between MGO and innate immune cells in relation to T2D.

Chapter 2 reviewed the formation and metabolism of MGO, the direct bactericidal effects of MGO, and the link between MGO and immune cell activation as potential mediator during host defence. Our current knowledge regarding the effects of MGO on immune cells mainly involve the induction of inflammation, apoptosis, and suppression of phagocytosis in neutrophils, monocytes and macrophages, and inhibition of lymphocyte activity. However, these effects of MGO remain debatable due to several reasons: 1) differences between endogenously produced MGO (i.e., within cells) and exogenously administrated MGO, 2) the use of unpurified MGO in most published studies and 3) the use of different concentrations of MGO and different incubation times in the different experimental studies.

In **Chapter 3**, we investigated whether postprandial levels of MGO in plasma and tissues originates from exogenous glucose and whether this increased plasma MGO concentration leads to a fast formation of MGO-derived advanced glycation endproducts (AGEs). A glucose tolerance test (GTT) with universally labelled D(+)^{13}C glucose was performed in healthy humans (oral (O)GTT) and in C57BL/6J mice (intraperitoneal (IP)GTT). We demonstrated that the newly formed MGO in human plasma during the OGTT is completely derived from exogenous glucose. Moreover, a fast formation of protein-bound MGO-derived AGEs during the OGTT was observed. In mice, we confirmed that the formation of postprandial MGO is derived from exogenous glucose in plasma, and we also

showed that the increased MGO in pancreas, spleen, kidney, subcutaneous and visceral adipose tissue (SAT and VAT), liver, and skeletal muscle during the IPGTT originates from exogenous glucose.

In **Chapter 4**, we evaluated MGO levels in circulating cells and investigated whether MGO formation in these cells during a glucose tolerance test originates from exogenous glucose, and whether obesity affects glucose-derived MGO formation. OGTT was performed in 19 abdominally obese individuals and IPGTT was performed in both C57BL/6J and db/db mice, with universally labelled D(+)^{13}C glucose. We found that MGO is present in extremely high concentrations in circulating immune cells compared to plasma, and increases during a GTT. Obesity increases exogenous glucose-derived MGO formation during the GTT in plasma, circulating immune cells, as well as in pancreas, liver, spleen, kidney, VAT, and SAT, but decreases MGO formation in erythrocytes. The lower levels of postprandial MGO in RBCs of db/db mice are most likely due to their low expression of glucose transporter GLUT1 compared to lean mice. The MGO stress in the postprandial phase may contribute to the detrimental effects and long-term complications due to postprandial glucose spikes.

In **Chapter 5**, we assessed the contribution of glycolysis in the exogenous glucose-derived MGO formation in mice during an IPGTT. 2 Deoxyglucose (2DG), which blocks glycolysis, was used in combination with universally labelled D(+)^{13}C glucose during the IPGTT in C57BL/6J mice. We found that glycolysis contributes to exogenous glucose-derived MGO formation in liver, skeletal muscle, SAT, and bone marrow, but not in blood cells, or in pancreas, spleen, kidney, and VAT. The postprandial MGO formation in plasma, blood cells, or in pancreas, spleen, kidney, and VAT is possibly formed spontaneously from glucose or is due to a direct uptake. Although a further validation is required, the findings in this chapter provide new insights into potential pathways for postprandial MGO formation.

Chapter 6 studied the effects of MGO on immune cell counts and activation, as well as on trained immunity. To investigate this, C57BL/6J mice received either a single intravenous injection of MGO, or a long-term exposure of MGO as supplemented in drinking water. Neither treatment showed robust effects on immune cell count or activation. Interestingly, a single high-dose MGO injection, but not long-term MGO intake via drinking water, enhanced LPS-induced nitric oxide production and proinflammatory gene expression in BMDMs from these

mice, suggesting the induction of trained immunity. We confirmed this innate training effect of MGO in primary human monocytes, where trained immunity was induced by β-glucan, and MGO formation was enhanced during training. This β-glucan-induced trained immunity was blunted by the MGO scavenger aminoguanidine during the training, while it was enhanced by adding additional MGO. These findings indicate that MGO does not directly affect immune cell counts or activation, but play a potential role in trained immunity.

To connect the experimental findings with clinical data, in **Chapter 7**, we investigated whether fasting or post-glucose-load plasma MGO concentrations are associated with circulating immune cell counts and activation in a large human cohort study. We included 696 participants (54% normal glucose tolerance, 13% prediabetes, and 33% T2D) from The Maastricht Study. Associations were analysed with multiple linear regression adjusted for age, sex, body mass index, education, smoking, systolic blood pressure, medication use, and glucose metabolism status. We found that higher fasting plasma MGO concentrations were significantly associated with higher numbers of intermediate and non-classical monocytes, while with lower activation for intermediate monocytes. No consistent associations were shown for post-OGTT plasma MGO levels with either immune cell counts or activation. These findings support a potential interaction between plasma MGO and circulating intermediate monocytes, as a possible contributor to the increased risk of cardiovascular disease in individuals with T2D.

Postprandial methylglyoxal formation in health and obesity

Chronic hyperglycaemia is linked to the development of diabetic cardiovascular complications[1,2]. Several studies have suggested that especially postprandial glucose excursions play a role in the increased risk for cardiovascular disease in people with T2D[3-9]. A potential mechanism behind the glucose-induced cardiovascular risk is the formation of MGO. We have previously shown that plasma MGO concentrations rapidly increased during an OGTT and a mixed meal test, with a higher increase in individuals with T2D[10,11]. Repeated episodes of elevated MGO plasma concentration may lead to MGO stress and may explain the detrimental effects of postprandial glucose spikes[9,12].

Previous data in humans showed a link between increased levels of postprandial glucose and MGO in plasma. It was, however, unknown what the contribution was of exogenous glucose to the MGO increase. Whether postprandial MGO formation directly originates from exogenous glucose during a glucose tolerance test was studied in **Chapter 3 and 4**. The use of universally labelled D(+)^{13}C glucose allowed us to evaluate the concentrations of $^{13}C_3$ MGO that is formed from exogenous glucose. We found that under healthy conditions, postprandial MGO formation increased in plasma in both humans and mice, and this increase of MGO was completely derived from exogenous glucose. The formation of MGO was followed by a fast formation of MGO-derived AGEs. In addition to plasma, newly formed MGO in blood cells as well as in mouse tissues such as pancreas, liver, spleen, kidney, skeletal muscle, SAT and VAT also originated from exogenous glucose. As described in **Chapter 2**, glycolytic consumption of glucose is considered to be the major source of endogenously formed MGO, via the non-enzymatic degradation of the glucotrioses glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), which contributes about 90% to the total amount of MGO. Other pathways for MGO formation in relation to glucose include auto-oxidation of glucose and degradation of glycated proteins. Therefore, in **Chapter 5**, we further assessed the contribution of glycolysis to the exogenous glucose-derived MGO formation in blood and tissues in mice during an IPGTT. 2DG is an inhibitor of the first two enzymes in the glycolytic pathway - phosphoglucose isomerase and hexokinase[13,14] and was used to block glycolysis. Interestingly, we found a significant contribution of glycolysis to postprandial MGO formation in liver, skeletal muscle, SAT, and bone marrow, but not in plasma and other tissues. As elevated formation of exogenous glucose-derived MGO in plasma, blood cells and in other tissues (**Chapter 3 and 4)** was either not changed or even tended to be higher with 2DG administration**,** we speculated that this increased postprandial MGO is produced spontaneously from glucose or from a direct uptake. *Ex vivo* experiments indeed demonstrated a spontaneous formation of MGO from glucose in human plasma, as well as in bovine serum albumin (BSA). This spontaneously formed MGO seemed to balance the amount of MGO that reacted rapidly with proteins. Based on these findings, a spontaneous formation of MGO can be expected also in blood cells as well as in tissues, where a glucose spike occurred. In addition, a direct MGO uptake from plasma in tissues cannot be fully excluded, although passive transport of MGO from plasma to immune cells are not likely to happen due to the very high intracellular MGO levels in immune cells compared to MGO concentrations in plasma. For tissues where MGO levels did not change after

2DG injection (pancreas, spleen, and VAT), glycolysis as well as spontaneous MGO formation and a direct MGO uptake seem to take place simultaneously in the postprandial phase. Future investigations are needed to validate these findings. Nevertheless, these data provide new insights into the pathways for exogenous glucose-derived MGO formation in the postprandial phase.

Postprandial MGO accumulation in plasma was previously shown to further increased in individuals with impaired glucose metabolism and T2D[10,11]. Weight loss interventions reduced postprandial plasma MGO levels[10,15]. We additionally investigated the effects of obesity on blood cell and tissue MGO formation from exogenous glucose (**Chapter 4).** Our findings demonstrated again that exogenous glucose directly contribute to MGO formation in blood fractions and in several tissues, and with higher levels of exogenous glucose-derived MGO in db/db mice, except for MGO in erythrocytes. A possible explanation of the latter is the decreased expression of the main glucose transporter GLUT1 in erythrocytes of db/db mice. Indeed, a significant lower GLUT1 expression levels on RBC membrane were shown in individuals with T2D[16]. These data together support a potential role of abnormal postprandial MGO spikes in obesity and T2D, which may further contribute to the development of cardiometabolic complications. In order to better target MGO and reduce MGO stress in obesity and T2D, the exact pathways involved in postprandial MGO formation under pathological conditions need to be investigated.

Quantification of methylglyoxal concentrations

Concentrations of MGO in plasma, blood cells, and in tissues as described in **Chapter 3, 4, and 5**, were quantified using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)[17]. Several previous studies have also evaluated MGO concentrations in whole blood, plasma, erythrocytes, and in livers. However, because of different approaches of sample preparation and different ways of measurement, mixed results were presented in humans and animals[18-23]. Thornalley et al. pointed out that peroxidase activity in physiological samples is a potential interference in MGO measurement[24]. We therefore blocked peroxidase activity in the MGO measurement to avoid overestimation of MGO quantification. To our surprise, we showed that intracellular MGO concentrations in circulating granulocytes, monocytes, and lymphocytes are extremely high, which are 1000-fold higher than erythrocytes and 20,000-fold higher than plasma (**Chapter 4**). Similar findings were also shown in C57BL/6J mice. In addition, we also measured high intracellular

concentrations MGO in cultured endothelial cells. This is in contrast with published data about low concentrations of free MGO in endothelial cells[18]. However, it is important to note that MGO data, as measured in our study, are based on total MGO, which is a combination of free and protein-bound MGO. We found that the free MGO in circulating immune cells accounts for less than 4% of total MGO and similar low free MGO was also shown in endothelial cells. Therefore, to better compare data between different studies, differences in sampling and sample preparations and measurements of MGO should not be ignored.

Role of methylglyoxal on immune cells

Potential effects of MGO on immune cells such as induction of inflammation, apoptosis, and suppression of phagocytosis in neutrophils, monocytes and macrophages have been extensively described in **Chapter 2**. However, due to different culture conditions in different experimental settings and the contamination of MGO batches as used in different studies, results are not very consistent and controversial. Our studies as described in **Chapter 6 and 7** involve both experimental and clinical approaches, which will be discussed separately.

Experimental approach

We studied the direct effects of MGO on immune cell counts and activation and the involvement of MGO in trained immunity via both *in vivo* and *in vitro* experiments (in **Chapter 6)**. A single spike of MGO in healthy mice did not directly affect immune cell counts and activation in blood and in the liver, as well as in several other tissues. In line with these findings, incubation of bone marrow-derived macrophages (BMDM) or human monocyte-derived macrophages with MGO *in vitro* under several different experimental conditions, also failed to induce activation. There are several possible explanations for these null-findings. MGO may react rapidly with proteins in the culture medium, or MGO may be detoxified by the glyoxalase system. Another possibility is that the concentrations of MGO used in these *in vitro* studies is not high enough to affect these immune cells due to the high intracellular MGO levels. Indeed, levels of the exogenously added MGO were much lower than that of the intracellular stored MGO. In addition to the short-term spikes of MGO, long-term exposure of mice to MGO, as supplied in drinking water, also showed limited

direct effects on immune cell counts and activation. Although we showed increased MGO concentrations in plasma, we found, also to our surprise, decreased gene expression of proinflammatory markers. However, this was in agreement with a cohort study in humans where a long-term higher intake of dietary MGO was associated with higher MGO concentrations in plasma but with less low-grade inflammation[25,26]. These data indicate that under healthy conditions, a spike or chronic administration of exogenous MGO do not directly affect immune cell numbers and inflammation. However, MGO may affect adhesion and migration of immune cells in disease states[27].

In addition to the direct inflammatory effect, trained immunity (long-term memory of innate immune cells) is also related to activation of immune cells. We confirmed a priming effect of MGO in BMDMs from high-dose MGO spiked mice as well as in a β-glucan-induced model of human trained immunity. Although the mechanisms behind MGO-induced trained immunity remains unclear, a potential pathway may be via epigenetic modifications of MGO. MGO has been indicated to affect epigenetics via Set7-induced monomethylation of histone 3 lysine 4 (H3K4me1), in nuclear factor-κB (NF-κB) signalling[28,29]. Moreover, H3K4me1 has been involved in the induction of trained immunity[30]. It would be of interest to further explore this potential mechanism in future studies.

Clinical approach

In a population-based cohort study, we investigated the associations between fasting and post-OGTT plasma MGO concentrations with circulating immune cell numbers and activation (**Chapter 7)**. We found a significant association between higher fasting plasma MGO levels and more intermediate and non-classical monocytes, as well as less activation of intermediate monocytes. Stratified analyses suggested that these associations with cell counts appear only in participants with T2D. Increased numbers of circulating monocyte subsets, especially intermediate and non-classical monocytes have been linked with clinical outcomes of cardiovascular diseases, such as intima-media thickness[31,32], coronary plaque rupture[33,34], unstable angina[35], adhesion mediated by vascular cell adhesion molecule-1[36], coronary artery calcification[37], restenosis after peripheral percutaneous transluminal angioplasty[38], as well as cardiovascular events prediction[39-41]. The lower activation of intermediate monocytes may reflect exhaustion of cells, as induced by long-term exposure to high levels of plasma MGO. In line with this speculation, a previous study in individuals with T2D showed an impaired inflammatory response of monocytes,

as reflected by reduced TNF secretion and downregulation of CD11b expression, in response to LPS challenge[42]. Interestingly, these findings also seem to be in line with our experimental findings in **Chapter 6**, where long-term MGO administration in drinking water in mice led to an increase in plasma MGO levels, and a slight suppression in BMDM responsiveness, as reflected by reduced nitrite oxide production. Therefore, these findings support an interaction between plasma MGO and circulating intermediate monocytes, as a potential risk factor of cardiovascular disease in people with T2D.

Conclusion and perspectives

In this thesis, we combined animal studies, *in vitro* studies, and a large cohort study. We identified an interplay between MGO and innate immune cells, which may become a vicious cycle under type 2 diabetic conditions and this may further contribute to the development of diabetic macrovascular complications (Figure 8.1). In addition, this thesis provides important evidence for research in postprandial MGO formation under healthy and disease conditions and we showed for the first time an association between plasma MGO levels and immune cell counts and activation in a population-based cohort, as well as the involvement of MGO in trained immunity.

Future research on interactions between MGO and immune cells are still needed. Although we found that postprandial MGO formation is derived from exogenous glucose and partly dependent on glycolysis, the complex communications between different organs and between organs and circulation remain to be investigated. To get a better insight of postprandial MGO changes, levels of postprandial glucose, as well as levels of other compounds involved in the metabolic pathways of MGO should also be assessed. Evaluation of MGO detoxification, such as measurement by its endproduct D-lactate, is also of importance. In addition, this thesis identified an important role of MGO in the induction of trained immunity, as a possible mechanism of hyperglycaemia-induced cardiovascular risk. More work needs to be done in the future regarding the underlying mechanisms, which would be critical for targeting MGO in T2D and its vascular complications. With respect to the population-based cohort studies, our findings based on The Maastricht Study demonstrated an association between plasma MGO and circulating immune cells and indicated a potential role in the aetiology of cardiovascular disease in people with T2D.

These findings should be validated in other cohort studies and with a larger sample size.

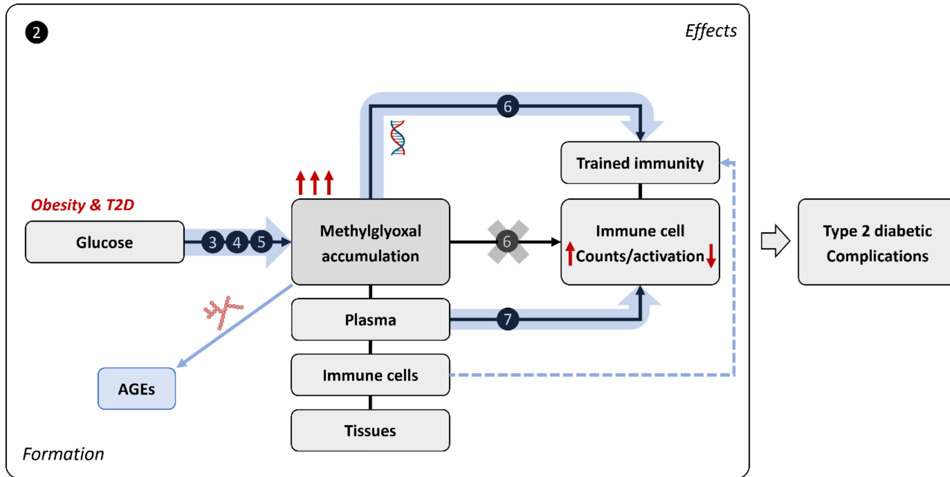

Figure 8.1 **Schematic overview of the main findings in this thesis.** Numbers indicate thesis chapters. Black solid arrows represent relations that we hypothesized and investigated. Blue solid lines represent relations found in the current thesis. Blue dashed lines represent potential relations. The formation of MGO and the link with inflammatory activation of immune cells, in relation to diseases.is reviewed in chapter 2. Postprandial methylglyoxal (MGO) formation in plasma, immune cells, and in tissues is derived from exogenous glucose, and followed by an increase in AGEs formation via reaction with proteins. Exogenous glucose-derived MGO formation is partly dependent on glycolysis. Obesity and T2D further increase postprandial MGO formation (chapter 3, 4, and 5). Exogenous MGO administration does not directly affect immune cell counts or activation, but is involved in the induction of trained immunity, possibly via epigenetic modifications. Increased endogenous formation of MGO may also contribute to trained immunity induction (chapter 6). Human cohort study showed an association of plasma MGO levels with higher immune cell counts, while with lower immune cell activation (chapter 7). *Abbreviations: T2D, type 2 diabetes; AGEs, advanced glycation endproducts.*

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Chapter 9

Valorisation addendum

Valorisation addendum

The burden of type 2 diabetes (T2D) is largely dependent on its cardiovascular complications. Current findings in this thesis implicate a role of methylglyoxal (MGO) and its interaction with immune cells in the development of cardiovascular disease. This chapter discusses the possibilities of MGO in immune cells as a potential risk marker and treatment target for T2D and cardiovascular disease, as well as potential treatment strategies.

Evaluation of methylglyoxal concentrations

The use of ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) allowed us to evaluate the precise concentrations of MGO in plasma, cells, and in different tissues. Universally labelled (D)+^{13}C glucose used in both human and mouse studies for the glucose tolerance test enabled us to evaluate the formation of MGO that was derived from exogenous glucose. We found that exogenous glucose during a glucose tolerance test directly contribute to postprandial MGO formation in blood and in tissues and that MGO formation is enhanced in obesity and T2D. These findings imply that postprandial MGO may be a potential marker for diabetes and its related diseases. Moreover, we showed for the first time that MGO is present in extremely high concentrations in circulating immune cells as compared to plasma. These data suggest an important role for intracellular formation of MGO rather than exogenously added MGO. Researchers should take this into account in future experimental design.

Overall, these findings provide important insights into the changes of postprandial MGO in the body in healthy and pathological conditions, and also imply that MGO in circulating immune cells deserve more attention in future research in the field of glycation in relation to T2D and its related complications.

Methylglyoxal as a treatment target

This thesis used a strong combination of animal studies, in vitro studies, and a large cohort study and identified MGO as a treatment target of cardiovascular disease, based on its effects on immune cells. We demonstrated in experimental studies that excess MGO may trigger trained immunity, i.e. a potential contributor to the high risk of cardiovascular disease in people with T2D. Under hyperglycaemic condition, trained immunity is induced in monocytes/ macrophages and promotes atherosclerosis[1]. Accumulation in immune cells of

MGO may lead to epigenetic changes and drive this hyperglycaemia-induced trained immunity. Although more measurements are needed regarding the epigenetic modifications, our findings suggested that MGO formation in innate immune cells is a potential target for the prevention of cardiovascular disease. Our investigation based on a population-based cohort (The Maastricht Study) also showed significant associations between fasting plasma MGO concentrations and higher numbers of circulating intermediate and non-classical monocytes, as well as intermediate monocyte exhaustion. Increased numbers of these cells are particularly relevant for the clinical outcomes of cardiovascular disease[2]. Exhausted monocytes that exposed to high levels of MGO may result in ineffective host defence, wound healing, and tissue repairment. These findings further support the need of targeting MGO in the treatment of diabetic vascular complications.

Potential future treatment strategies

There are several potential treatment strategies regarding MGO stress in T2D. The first is to achieve glycaemic control. Glucose-lowering drugs such as metformin, sulfonylurea, meglitinides, and thiazolidinediones are already used in the clinics[3]. Indeed, the use of metformin is associated with a reduction of MGO[4]. In addition, intensive lifestyle intervention such as a balanced diet and exercise, accompanied by clinically significant weight loss, also have been shown to improve glycaemic control and we previously demonstrated that weight loss is indeed associated with a reduction of MGO[5]. The second way to reduce MGO stress is a direct quenching of MGO. Pyridoxamine, a vitamin B6 analogue, is identified as an anti-glycating agent and has been shown to reduce MGO formation in high-fat diet-induced obese mice[6] and in a recently finished clinical trial in obese individuals[7]. In a clinical trial conducted at our department, we have showed that pyridoxamine has a favourable safety profile with no adverse effects[8]. In addition to pyridoxamine, we also demonstrated that quercetin is able to reduce the levels of MGO with 10%[9]. Third, enhancing GLO1 expression and activity to promote MGO metabolism can also help to reduce MGO stress. Cruciferous vegetables, which contain phenethyl isothiocyanate and sulforaphane, can be an option to stimulate GLO1[10] and to reduce the levels of MGO. In addition, *trans*-resveratrol and hesperetin coformulation, has recently been discovered as a GLO1 inducer, which significantly decreased plasma MGO levels in highly overweight subjects[11]. Future research may also consider to develop drugs that can be used after each meal to control postprandial endogenous production of MGO.

Methylglyoxal in immune cells as a potential risk marker

MGO in immune cells may serve as a potential risk marker for cardiovascular disease. Intracellular MGO levels in immune cells can be measured by 1) UPLC-MS/MS, 2) flow cytometry with a fluorescent MGO sensor probe, or 3) autofluorescence. Exact concentrations of MGO in total leukocytes can be directly analysed with UPLC-MS/MS and the combination with fluorescence activated cell sorting further enable the detection of MGO in specific immune cells. An alternative way to quantify MGO in circulating immune cells is with the use of the fluorescent sensor MBO (methyl diaminobenzene-BODIPY)[12], in combination with flow cytometry. Fluorescent intensities of the MBO probe reflect the relative levels of MGO in different immune cells. The strength of using UPLC-MS/MS is that the results are quantitative, and the detection of MGO can be achieved in both fresh and frozen samples. The use of flow cytometry requires fresh blood, is more efficient and faster, and is cheaper, but is semi-quantitative. In addition to the direct quantifications of MGO, we recently developed a novel way of measuring cellular autofluorescence with full spectrum cytometry. Autofluorescence is thought to reflect levels of MGO-derived advanced glycation endproducts, which may also be an option to indirectly detect MGO. Based on the availability of techniques in the laboratory to detect MGO, a choice can be made to study MGO in immune cells as a potential risk marker for cardiovascular disease.

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Appendices

Acknowledgements
Curriculum vitae
Scientific output

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Curriculum vitae

Xiaodi Zhang was born on December 4th, 1992 in Jilin, China. In 2011, she graduated from high school and enrolled in Jilin University, majoring in Biotechnology (Zoology). During her bachelor's study, the laboratory experience stimulated her interest in life science research. In 2015, she obtained her bachelor's degree and went on to pursue a master's degree in Biochemistry and Molecular Biology at Jilin University. During her master's study, she participated in several research projects, and her thesis focused on the effects of Sini Decoction (traditional Chinese medicine) on gut microbiota in mice with colorectal cancer. She received her master's degree in 2018, and in the same year, she was awarded a national grant from the China Scholarship Council for a 4-year PhD project. She conducted her PhD at the department of Internal Medicine and Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, under supervision of Prof. Dr. Casper Schalkwijk and Dr. Kristiaan Wouters. During her PhD, she investigated the formation of methylglyoxal and its interaction with immune cells in obesity and type 2 diabetes. In March 2023, she was awarded an Alexander von Humboldt Foundation research fellowship for postdocs, for a period of 24 months. After completing her doctorate, she will start her postdoctoral research at Institute for Molecular Cardiovascular Research (IMCAR), RWTH Aachen University. Her research will focus on the effect of (methylglyoxal-modified) high-density lipoprotein on trained immunity and atherosclerosis-related processes.

Scientific output

List of publications

Zhang X, Scheijen JLJM, Stehouwer CD, Wouters K, Schalkwijk CG. Increased methylglyoxal formation in plasma and tissues during a glucose tolerance test is derived from exogenous glucose. Clinical Science. 2023; 137(8): 697-706.

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Submitted

Zhang X, van Greevenbroek MMJ, Scheijen JLJM, Eussen SJPM, Kelly J, Stehouwer CDA, Schalkwijk CG*, Wouters K*. Fasting plasma methylglyoxal concentrations are associated with higher numbers of circulating intermediate and non-classical monocytes but with lower activation of intermediate monocytes: The Maastricht Study.

Oral presentations

Annual Dutch Diabetes Research Meeting 2022 (Wageningen, the Netherlands): Increased methylglyoxal formation in plasma and tissues during a glucose tolerance test originates from exogenous glucose and is enhanced in diabetes.

58th Annual Meeting of the European Association for the Study of Diabetes 2022 (Stockholm, Sweden): Increased methylglyoxal formation in plasma and tissues during a glucose tolerance test originates from exogenous glucose and is enhanced in diabetes.

57th Annual Meeting of the European Association for the Study of Diabetes 2021 (virtual): The postprandial methylglyoxal formation during an oral glucose tolerance test is derived from exogenous glucose.
56th Annual Meeting of the European Association for the Study of Diabetes 2020 (virtual): The glycolytic by-product methylglyoxal is present in immune cells and may affect their recruitment.

Annual Dutch Diabetes Research Meeting 2020 (virtual): The glycolytic by-product methylglyoxal is present in immune cells and may affect their recruitment.

Annual Dutch Diabetes Research Meeting 2019 (Wageningen, the Netherlands): The glycolytic by-product methylglyoxal is present in monocytes and granulocytes and has pro-inflammatory properties.

Poster presentations

International Maillard Reaction Society – 14 (IMARS-14, virtual): The postprandial methylglyoxal formation during an oral glucose tolerance test is derived from exogenous glucose.

Digital annual meeting NVVI 2020 (Dutch Society for Immunology): The glycolytic by-product methylglyoxal is present in immune cells and may affect their recruitment.

55th Annual Meeting of the European Association for the Study of Diabetes 2019 (Barcelona, Spain): The glycolytic by-product methylglyoxal is present in monocytes and granulocytes and has pro-inflammatory properties.

Awards

Annual Dutch Diabetes Research Meeting 2022 (Wageningen, the Netherlands): best abstract award.