Plasma Methylglyoxal Levels Are Associated With Amputations and Mortality in Severe Limb Ischemia Patients With and Without Diabetes

Nordin M.J. Hanssen,[1,2] Martin Teraa,[3] Jean L.J.M. Scheijen,[1] Marjo Van de Waarenburg,[1] Hendrik Gremmels,[4] Coen D.A. Stehouwer,[1] Marianne C. Verhaar,[4] and Casper G. Schalkwijk[1]

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OBJECTIVE

Diabetes is a risk factor for severe limb ischemia (SLI), a condition associated with high mortality, morbidity, and limb loss. The reactive glucose-derived dicarbonyl methylglyoxal (MGO) is a major precursor for advanced glycation end products (AGEs) and a potential driver of cardiovascular disease. We investigated whether plasma MGO levels are associated with poor outcomes in SLI.

RESEARCH DESIGN AND METHODS

We measured plasma levels of MGO, free AGEs, and D-lactate, the detoxification end product of MGO, with ultraperformance liquid chromatography–tandem mass spectrometry at baseline in 160 patients (64.8 ± 13.3 years, 67.5% male, 37.5% with diabetes) with no-option SLI and recorded major adverse outcomes (n = 86, comprising n = 53 deaths and n = 49 amputations [first event counted]) over the 5-year follow-up. Data were analyzed with linear or Cox regression, after Ln-transformation of the independent variables, adjusted for sex, age, trial arm, diabetes, estimated glomerular filtration rate, systolic blood pressure, cholesterol levels, and BMI. Associations are reported per 1 SD plasma marker.

RESULTS

Higher plasma MGO levels were associated with more adverse outcomes (relative risk 1.44; 95% CI 1.11–1.86) and amputations separately (1.55; 1.13–2.21). We observed a similar but weaker trend for mortality (1.28; 0.93–1.77). The MGO-derived AGE N^{ε}-(carboxyethyl)lysine was also associated with more adverse outcomes (1.46; 1.00–2.15) and amputations (1.71; 1.04–2.79). D-Lactate was not associated with adverse incident outcomes. Higher plasma MGO levels were also associated with more inflammation and white blood cells and fewer progenitor cells.

CONCLUSIONS

Plasma MGO levels are associated with adverse outcomes in SLI. Future studies should investigate whether MGO-targeting therapies improve outcomes in SLI.

[1]Department of Internal Medicine, Cardiovascular Research Institute Maastricht (CARIM) School for Cardiovascular Diseases, Maastricht University, Maastricht, the Netherlands
[2]Diabetes Center, Department of Vascular and Internal Medicine, Amsterdam University Medical Centre, Amsterdam, the Netherlands
[3]Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, the Netherlands
[4]Department of Nephrology and Hypertension, University Medical Center Utrecht, Utrecht, the Netherlands

Corresponding author: Nordin M.J. Hanssen, nmj.hanssen@maastrichtuniversity.nl

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Diabetes is a major risk factor for severe limb ischemia (SLI), the most advanced stage of peripheral arterial disease (PAD) (1). Despite advances in less invasive revascularization techniques and cardiovascular risk management, SLI is still a condition associated with very high mortality, morbidity, limb loss, and health care costs (1). Therefore, there is a need for new medical approaches to reduce adverse outcomes in SLI.

A potential therapeutic target to reduce adverse outcomes in SLI is the accumulation of dicarbonyl compounds such as methylglyoxal (MGO). These reactive glucose metabolites lead to the rapid formation of advanced glycation end products (AGEs), such as the major AGE N^{ε}-(carboxymethyl)lysine (CML) and the MGO-derived lysine modification N^{ε}-(carboxyethyl)lysine (CEL), and arginine modification 5-hydro-5-methylimidazolone (MG-H1) (2). MGO has been identified as the most reactive dicarbonyl compound (2), and higher plasma MGO levels are associated with chronic kidney disease and incident cardiovascular disease in type 1 and type 2 diabetes (3,4). Increased formation of MGO is linked to hyperglycemia (5), inflammation, oxidative stress, and hypoxia (6,7), all important factors determining adverse outcomes in SLI (8,9). In turn, MGO accumulation contributes to cellular dysfunction and cell death through formation of reactive oxygen species and protein and DNA modifications (2).

MGO can be detoxified by the glyoxalase pathway into D-lactate, in which glyoxalase 1 (Glo1) is the rate-limiting enzyme. Glo1 expression is lower in ruptured plaque segments and in diabetic chronic kidney disease (6,10). Whether D-lactate is associated with adverse outcomes in SLI is unknown.

An important driver of adverse outcomes in SLI is an imbalance between inflammatory cytokines, circulating cells such as neutrophils and monocytes, and reduced repair by circulating progenitor cells, leading to increased risk of adverse outcomes through enhanced tissue destruction with impaired repair (11,12). MGO levels are high in circulating cells (13), and MGO-derived AGEs are abundant in plaque macrophages (6), but whether plasma MGO levels are associated with circulating monocytes, neutrophils, or circulating and bone marrow (BM) progenitor cells is unknown.

We hypothesize that accumulation of dicarbonyl compounds drives poor outcomes such as limb loss and mortality in SLI. Therefore, we measured plasma MGO, free fractions of the AGEs CML and CEL, MG-H1, and the major MGO metabolite D-lactate and studied associations with adverse outcomes (mortality and amputations) in 160 patients with no-option SLI, many of whom had diabetes, who were included in the Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-arterial Supplementation (JUVENTAS) trial (9). In addition, we investigated cross-sectional associations of MGO and the other glycation markers with major drivers of poor outcome in SLI: inflammation and circulating monocytes, neutrophils, thrombocytes, and progenitor cells (9,11,14). Finally, we also investigated whether associations with major adverse outcomes differed for the presence of diabetes.

RESEARCH DESIGN AND METHODS

Study Population

The study population of this prospective cohort study consisted of 160 patients included in the JUVENTAS trial (14), a single-center double-blind randomized placebo-controlled trial investigating repetitive intra-arterial infusion of autologous BM mononuclear cells for the treatment of SLI without revascularization options. Initial characterization of study covariates and trial results of the primary analysis were performed by standard operating procedures and have been published elsewhere (9). Presence of diabetes was assessed with a questionnaire and by review of the medication records. Plasma inflammatory markers, including interleukin 6 (IL-6) and C-reactive protein (CRP), were measured with a multiple cytokine assay (Bio-Rad, Hercules, CA) (8).

Study inclusion was from September 2006 until June 2012. Inclusion criteria for the trial were severe infrapopliteal atherosclerosis, Fontaine grade IIB–IV, and ineligibility for surgical intervention. Exclusion criteria were a history of neoplasm or malignancy in the past 10 years, concomitant disease with a life expectancy of <1 year, inability to obtain sufficient BM aspirate, known infection with HIV, hepatitis B or C virus, and an expected inability to complete follow-up. Patients were randomized 1:1 to receive three intra-arterial infusions of autologous BM mononuclear cells or placebo into the common femoral artery of the affected limb.

Adverse Outcomes

The primary outcome of the initial trial was the incidence of major amputation, defined as amputation through or above the ankle joint, at 6 months after inclusion. For the current study, we extended the follow-up until mid-2017, using 5-year amputation-free survival as the primary outcome. If an amputation occurred before death, this event was counted for this outcome. Outcomes were collected using patient medical records or by contacting patients by telephone.

Ethics Statement

The study was approved by the UMC Utrecht (Utrecht, the Netherlands) Medical Ethics Committee (no. 06/030) and was conducted in accordance with the Declaration of Helsinki. All included patients provided written and verbal informed consent before inclusion.

Measurement of Plasma Dicarbonyls, Free AGEs, and D-Lactate

Peripheral blood samples were obtained before the BM harvesting and intra-arterial infusions by venipuncture of the antecubital vein and stored at -80°C until further analyses. We used ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) to measure plasma levels of MGO (15) as well as plasma free CML, CEL, and MG-H1 (16) and D-lactate levels, as previously described (17). All inter- and intrarun coefficients of variation were $<10\%$.

White Blood Cells and Thrombocytes

Circulating white blood cells, neutrophils, monocytes, lymphocytes, and thrombocytes were measured with an automated hematology analyzer (Cell-Dyn 1800; Abbott).

Flow Cytometry of Blood and BM Progenitor Cells

Before treatment allocation, ~100 mL of BM was harvested from the right iliac crest by an experienced hematologist under local anesthesia and conscious sedation. Flow cytometry on peripheral blood and BM was performed using lyse-and-wash protocols. Then, 100 μL of peripheral blood or BM was incubated

with an antibody panel, as described previously. Erythrocytes were lysed in an ammonium chloride buffer, and remaining cells were washed with PBS and analyzed by flow cytometry (FC 500; Beckman Coulter, Fullerton, CA). Flow cytometry data analysis data were analyzed using FlowJo 10.0.8 software (Treestar, Ashland, OR). To account for variations in acquisition occurring due to inconsistency in erythrocyte lysis, all cell numbers were corrected for the number of granulocytes, because this population can be most reliably identified on forward and side scatter. For the current analyses, we focused on $CD34^+$ and $CD133^+$ progenitor cells because only these were associated with adverse outcomes in this study in prior analyses (11).

Statistical Analysis

All analyses were performed with SPSS 20 for Windows (IBM, Armonk, NY). Plasma MGO, free AGEs, and D-lactate were Ln-transformed before further analyses to allow for calculation of Z scores because they showed a skewed distribution. Plasma CRP, IL-6, leukocytes, and thrombocytes were also Ln-transformed in analyses where they served as dependent variables. Linear regression models were used to investigate cross-sectional associations between Z scores of plasma glycation markers and continuous outcomes.

Cox proportional hazards regression models were applied to investigate associations between Z scores of the plasma glycation markers and adverse outcomes of mortality and/or amputation and mortality and amputations separately. All multivariable analyses were adjusted for sex, age, and study arm of the original trial (model 1). Further adjustments were performed for the presence of diabetes, BMI, systolic blood pressure, total cholesterol, current smoking, and estimated glomerular filtration rate (eGFR), except for analyses with decreased eGFR as the dependent variable (model 2). To investigate potential confounding by inflammation (because this influences circulating white blood cells), we adjusted associations between plasma MGO, free AGEs, or D-lactate levels and circulating white blood cell populations and thrombocytes for CRP (model 3). For our primary outcome, we investigated whether any of the associations differed by the presence of diabetes (presence of diabetes $\times$ plasma marker) or initial treatment assignment (treatment arm $\times$ plasma marker) by adding an interaction term to the model; for these analyses $P < 0.10$ was considered statistically significant. Missing values on any of the covariates (BMI, $n = 5$; systolic blood pressure, $n = 6$; smoking behavior, $n = 2$) were replaced with multiple imputation, and analyses were reported for the pooled estimate of five imputed data sets.

RESULTS

Table 1 summarizes the baseline characteristics of the JUVENTAS study patients. After a median follow-up of 4.3 years, an adverse event (mortality or amputation) occurred in 86 individuals. In total, 53 individuals died and 49 underwent an amputation. An amputation occurred in 16 individuals before death.

Associations Between Plasma MGO, Free AGEs, D-Lactate, and Adverse Outcomes (Death and/or Amputation)

Higher plasma MGO levels were associated with more adverse events after adjustment for sex, age, and study arm of the original JUVENTAS trial (Table 2, model 1). This association did not attenuate when we further adjusted for presence of diabetes, BMI, systolic blood

Table 1—Baseline characteristics of the JUVENTAS trial according to amputation-free survival at end of follow-up

	No event ($n = 74$)	Amputation or death ($n = 86$)
Age (years)	60.0 ± 12.0	69.1 ± 12.5
Sex (male)	56.9	75.6
Fasting glucose (mmol/L)	6.0 ± 1.4	6.9 ± 3.0
Diabetes	30.6	43.0
Insulin use	15.3	24.4
BMI (kg/m^2)	27.0 ± 4.0	25.9 ± 5.0
Total cholesterol (mmol/L)	4.4 ± 1.2	4.1 ± 1.1
HDL cholesterol (mmol/L)	1.3 ± 0.4	1.1 ± 0.4
Triglycerides (mmol/L)	1.5 (0.9–2.0)	1.5 (1.0–2.1)
Lipid-lowering medication use	86.1	82.6
ACE inhibitor use	33.3	44.2
Angiotensin receptor blocker use	22.2	20.9
Diuretic use	36.1	53.5
β-Blocker use	38.9	48.8
Antiplatelet medication use	68.1	70.9
Anticoagulation use	36.1	40.7
Leukocytes ($\times 10^9$/L)	7.9 (6.5–9.8)	8.6 (7.0–10.3)
Thrombocytes ($\times 10^9$/L)	283.5 (223.8–335.8)	279.5 (233.0–349.3)
Serum creatinine (μmol/L)	84.5 (72.3–93.8)	105.5 (76.0–144.3)
eGFR (mL/min/1.73 m^2)	76.8 (62.9–87.0)	62.0 (43.8–86.9)
eGFR <60 mL/min/1.73 m^2	19.4	44.2
Systolic blood pressure (mmHg)	131.3 ± 20.3	134.0 ± 18.4
Diastolic blood pressure (mmHg)	74.6 ± 10.3	79.0 ± 14.1
Smoking		
Never	9.7	17.9
Former	58.3	60.7
Current	31.9	21.4
Transcutaneous O_2 foot (mmHg)	43.5 ± 22.0	29.7 ± 21.0
Ankle-brachial index	0.5 (0.4–0.7)	0.5 (0.3–0.8)
CRP (mg/L)	4.2 (1.8–13.2)	8.7 (3.7–25.2)
MGO (nmol/L)	532.6 (491.1–611.9)	619.9 (526.7–723.3)
Free CML (nmol/L)	125.9 (96.6–170.1)	165.4 (124.4–304.6)
Free CEL (nmol/L)	57.7 (45.0–72.2)	88.6 (57.2–148.7)
Free MG-H1 (nmol/L)	238.7 (174.4–437.7)	364.3 (225.2–746.5)
D-Lactate (μmol/L)	10.1 (7.6–15.9)	11.2 (8.3–16.5)

Data are presented as means ± SD, medians (interquartile ranges), or percentages, as appropriate.

pressure, total cholesterol, current smoking, and eGFR (Table 2, model 2). When we analyzed death and amputation separately, we found that plasma MGO was associated with mortality (Table 2, model 1) but attenuated and lost its statistical significance after further adjustment (Table 2, model 2). Higher plasma MGO levels were associated with incident amputations (Table 2, models 1 and 2).

In line, higher free plasma levels of CEL, the MGO-derived AGE, were associated with death and/or amputations (Table 2, model 2) and incident amputations separately (Table 2, model 2). Plasma free CEL levels were associated with mortality in the basic model (Table 2, model 1), but this association was completely attenuated in the fully adjusted model (Table 2, model 2). In addition, free levels of the most abundant MGO modification, MG-H1 was associated with mortality (Table 2, model 1), although this association was attenuated slightly and lost statistical significance after additional adjustment (Table 2, model 2). Higher free plasma CML levels overall tended to be associated with more adverse events, but these associations were not significant (Table 2, models 1 and 2). Plasma D-lactate levels were not associated with mortality or amputation (Table 2, models 1 and 2).

Cross-sectional Associations Between Plasma MGO, Free AGEs, D-Lactate, and Plasma Markers of Inflammation

After adjustment for potential confounders, higher plasma MGO levels were associated with higher CRP and IL-6 levels (Table 3, model 2). Neither plasma levels of the free AGEs nor D-lactate were significantly associated with CRP or IL-6 (Table 3, model 2).

Cross-sectional Associations Between Plasma MGO, Free AGEs, D-Lactate, and Circulating White Blood Cells and Platelets

Plasma MGO levels were associated with higher total leukocyte counts and more circulating neutrophils, monocytes, and thrombocytes, but not lymphocytes (Table 4, models 1 and 2). After adjustment for potential confounders, the free plasma AGEs were not associated with any of the circulating cells (Table 4, models 1–3). Higher plasma D-lactate levels were associated with more circulating white blood cells, neutrophils, and monocytes, but not with lymphocytes or thrombocytes. When we adjusted these associations further for CRP, as a potential confounding marker of inflammation/infection, all associations remained virtually unaltered (Table 4, models 2 and 3).

Cross-sectional Associations Between Plasma MGO, Free AGEs, D-Lactate, and Progenitor Cells

Higher plasma MGO levels were consistently associated with fewer circulating and BM progenitor cells of both the $CD34^+$ and $CD133^+$ subset (Table 5, models 1 and 2). We observed a pattern of higher plasma free AGEs and lower progenitor cells, but these associations were overall no longer significant in the fully adjusted models (Table 5, model 2). We found no significant associations of D-lactate with circulating and BM progenitor cells.

Additional Analyses

Associations between higher plasma MGO levels and more adverse outcomes (death and/or amputation) were not materially altered and remained statistically significant after additional adjustment for CRP (relative risk 1.39; 95% CI 1.07–1.82) and IL-6 (relative risk 1.37; 95% CI 1.04–1.81). The association between higher MGO levels and more adverse outcomes also did not attenuate when we adjusted for any of the circulating white blood cells, platelets, or progenitor cells (data not shown). Although the presence of diabetes and higher plasma glucose levels were overall associated with higher plasma glycation markers (Supplementary Table 1), we found no evidence that the associations with adverse outcome consistently differed for the presence of diabetes (data not shown).

We did not find that the glycation markers differed consistently per original trial arm (data not shown).

Table 2—Associations between plasma dicarbonyls, free AGE, and D-lactate levels and 5-year outcomes of the JUVENTAS trial

	Model	Mortality and/or amputation	Mortality	Amputation
MGO	1	**1.34 (1.05–1.63)**	**1.47 (1.12–1.87)**	**1.33 (1.05–1.70)**
	2	**1.44 (1.11–1.86)**	1.28 (0.93–1.77)	**1.55 (1.13–2.21)**
Free CML	1	1.19 (0.96–1.47)	**1.59 (1.25–2.03)**	1.03 (0.76–1.39)
	2	1.33 (0.92–1.95)	1.52 (0.94–2.47)	1.33 (0.80–2.19)
Free CEL	1	1.21 (0.97–1.51)	**1.42 (1.10–1.84)**	1.09 (0.81–1.46)
	2	**1.46 (1.00–2.15)**	1.01 (0.62–1.63)	**1.71 (1.04–2.79)**
Free MG-H1	1	1.16 (0.93–1.46)	**1.65 (1.25–2.18)**	0.95 (0.70–1.30)
	2	1.23 (0.90–1.68)	1.51 (0.99–2.30)	1.12 (0.75–1.68)
D-Lactate	1	1.05 (0.86–1.28)	0.96 (0.74–1.25)	1.10 (0.84–1.44)
	2	1.03 (0.83–1.27)	1.06 (0.78–1.44)	1.01 (0.76–1.34)

Data were analyzed with Cox regression. HRs are presented per SD increase of Ln-normalized independent variable. Bold values are significant. Model 1 is adjusted for sex, age, and trial arm. Model 2 is further adjusted for BMI, presence of diabetes, smoking, eGFR, plasma cholesterol, and systolic blood pressure.

CONCLUSIONS

This study shows for the first time in individuals with SLI, many of whom had diabetes, that plasma MGO levels are associated with adverse events (death and/or amputations). In cross-sectional analysis, we found the MGO is associated with higher CRP and IL-6 levels; higher neutrophil, monocyte, and thrombocyte counts; and lower circulating and BM progenitor cells. However, we did not find that any of these drivers of SLI explained the association between plasma MGO levels and adverse outcomes. We found similar associations for free levels of CEL, the MGO-derived AGE, with adverse outcomes. Overall, we found no associations between the other dicarbonyls and D-lactate with adverse outcomes.

To our knowledge, only few studies have investigated the role of glycation in adverse outcomes of SLI, such as mortality or ulceration of the lower leg requiring amputation (18). Previously, skin autofluorescence, a marker of AGEs, has been reported to be associated with amputations in PAD (18). Our study is the first to investigate whether plasma dicarbonyls and D-lactate levels are associated with adverse outcomes in SLI. In

Table 3—Associations between plasma dicarbonyl, free AGE, and D-lactate levels and CRP and IL-6 levels

	Model	CRP	IL-6
MGO	1	**0.18 (0.02–0.33)**	**0.35 (0.20–0.49)**
	2	**0.18 (0.00–0.35)**	**0.34 (0.17–0.51)**
Free CML	1	0.00 (−0.19 to 0.20)	0.08 (−0.12 to 0.27)
	2	0.05 (−0.24 to 0.34)	0.06 (−0.22 to 0.34)
Free CEL	1	0.02 (−0.17 to 0.22)	0.14 (−0.05 to 0.33)
	2	0.06 (−0.25 to 0.36)	0.17 (−0.13 to 0.46)
Free MG-H1	1	0.04 (−0.15 to 0.23)	0.07 (−0.12 to 0.26)
	2	0.04 (−0.20 to 0.27)	0.05 (−0.18 to 0.27)
D-Lactate	1	0.11 (−0.05 to 0.27)	0.12 (−0.04 to 0.28)
	2	0.07 (−0.09 to 0.24)	0.08 (−0.08 to 0.24)

All data were analyzed with linear regression. Bold values are significant. β values are presented per SD increase of Ln-normalized independent variable per SD increase of the Ln-normalized outcome variables. All continuous outcome variables were Ln transformed. Model 1 is adjusted for sex, age and trial arm. Model 2 is further adjusted for BMI, presence of diabetes, smoking, eGFR, plasma cholesterol, and systolic blood pressure.

line with experimental studies that have identified MGO as the most reactive dicarbonyl (2), our current findings that plasma MGO levels are associated with adverse outcomes are also in line with our previous observations in large cohort studies on cardiovascular outcomes, including PAD, in type 1 and 2 diabetes (3,4). Our current study demonstrates that plasma MGO levels are also associated with adverse outcomes in individuals without diabetes.

This is, to our knowledge, the first study to investigate D-lactate levels with any adverse outcome. D-Lactate was weakly associated with MGO levels. Perhaps this weak association is because D-lactate levels may on the one hand be increased by high rates of MGO detoxification, while on the other hand they may be lowered by reduced Glo1 activity (19). Furthermore, D-lactate is also produced by certain gut bacteria (17). These factors combined may weaken associations between systemic MGO and D-lactate levels.

This study has an important hypothesis-generating function. We found that plasma MGO levels were associated with more circulating neutrophils, monocytes, and thrombocytes, while MGO was also associated with exhaustion of the BM progenitor cells, an independent risk marker for adverse outcome in SLI (11). The precise mechanisms of how MGO dysregulates cellular subpopulations of the BM in favor of more inflammatory cells and less progenitor cells is an exciting research area that is to our knowledge largely unexplored. Interestingly, a mouse study using the hind limb ischemia model for SLI found that AGE-modified albumin produced by macrophages disrupts progenitor cells in a receptor for AGEs (RAGE)-dependent mechanism, leading to poor outcome and more muscle loss (20). We indeed identified the macrophage as the principal cell of MGO-derived AGE accumulation in the atherosclerotic plaque (6), but whether this is a targetable disease mechanism in humans is not yet known. In addition, we did not find any obvious covariate that attenuated the association between plasma MGO levels and adverse outcome. Therefore, the main mechanism through which MGO causes adverse outcome in SLI remains unclear and may be due to the direct toxic effect of MGO on tissues.

The major strength of this study is its prospective design and a large sample containing a unique patient group, providing a large number of individuals reaching the primary end point after a 5-year follow-up. In addition, we used state-of-the-art equipment to measure plasma MGO and D-lactate.

However, this study also has a few limitations. Firstly, this study was originally designed as a clinical trial; therefore, we cannot fully rule out that the

Table 4—Associations between plasma dicarbonyls, free AGE, and D-lactate levels and circulating leukocytes and platelets

	Model	Total leukocytes	Neutrophils	Lymphocytes	Monocytes	Thrombocytes
MGO	1	**0.23 (0.07–0.38)**	**0.25 (0.10–0.40)**	−0.05 (−0.19 to 0.10)	**0.26 (0.11–0.41)**	**0.35 (0.21–0.49)**
	2	**0.28 (0.11–0.45)**	**0.26 (0.08–0.43)**	0.06 (−0.08 to 0.25)	**0.25 (0.08–0.41)**	**0.51 (0.36–0.66)**
	3	**0.28 (0.12–0.44)**	**0.25 (0.09–0.42)**	0.09 (−0.08 to 0.25)	**0.24 (0.08–0.41)**	**0.51 (0.36–0.66)**
Free CML	1	0.00 (−0.17 to 0.16)	0.08 (−0.08 to 0.25)	**−0.31 (−0.46 to −0.16)**	0.11 (−0.05 to 0.27)	**−0.16 (−0.32 to 0.00)**
	2	0.08 (−0.19 to 0.34)	0.12 (−0.15 to 0.39)	−0.20 (−0.44 to 0.04)	0.10 (−0.16 to 0.36)	−0.20 (−0.46 to 0.06)
	3	0.12 (−0.14 to 0.37)	0.16 (−0.10 to 0.42)	−0.20 (−0.44 to 0.04)	0.13 (−0.12 to 0.38)	−0.18 (−0.43 to 0.08)
Free CEL	1	0.02 (−0.15 to 0.19)	0.09 (−0.08 to 0.26)	**−0.24 (−0.40 to −0.08)**	0.12 (−0.05 to 0.28)	−0.11 (−0.28 to 0.05)
	2	0.17 (−0.11 to 0.44)	0.17 (−0.11 to 0.46)	−0.02 (−0.27 to 0.24)	0.12 (−0.15 to 0.39)	−0.02 (−0.29 to 0.25)
	3	0.16 (−0.10 to 0.43)	0.17 (−0.10 to 0.44)	−0.02 (−0.27 to 0.24)	0.12 (−0.15 to 0.38)	−0.02 (−0.29 to 0.24)
Free MG-H1	1	−0.02 (−0.18 to 0.15)	0.07 (−0.09 to 0.24)	**−0.27 (−0.42 to −0.12)**	0.08 (−0.08 to 0.24)	−0.14 (−0.30 to 0.02)
	2	0.03 (−0.19 to 0.24)	0.08 (−0.14 to 0.29)	−0.15 (−0.35 to 0.04)	0.06 (−0.15 to 0.27)	−0.10 (−0.31 to 0.11)
	3	0.06 (−0.15 to 0.26)	0.11 (−0.10 to 0.31)	−0.15 (−0.35 to 0.04)	0.08 (−0.12 to 0.29)	−0.08 (−0.29 to 0.12)
D-Lactate	1	**0.17 (0.01–0.32)**	**0.17 (0.01–0.34)**	0.11 (−0.04 to 0.26)	**0.17 (0.01–0.34)**	0.03 (−0.14 to 0.19)
	2	**0.19 (0.03–0.35)**	**0.20 (0.02–0.37)**	0.15 (−0.01 to 0.30)	**0.20 (0.02–0.37)**	0.05 (−0.12 to 0.22)
	3	**0.20 (0.04–0.35)**	**0.20 (0.03–0.36)**	0.15 (−0.01 to 0.30)	**0.20 (0.03–0.36)**	0.03 (−0.14 to 0.19)

All data were analyzed with linear regression. Bold values are significant. β values are presented per SD increase of Ln-normalized independent variable per SD increase of the Ln-transformed outcome variables. Model 1 is adjusted for sex, age, and trial arm. Model 2 is further adjusted for BMI, presence of diabetes, smoking, eGFR, plasma cholesterol, and systolic blood pressure. Model 3 is further adjusted for CRP as a continuous variable.

Table 5—Associations between plasma dicarbonyls, free AGE, and D-lactate levels and circulating and BM progenitor cells

	Model	Circulating $CD34^+$	BM $CD34^+$	Circulating $CD133^+$	BM $CD133^+$
MGO	1	**−0.20 (−0.36 to −0.05)**	**−0.35 (−0.49 to −0.20)**	−0.15 (−0.29 to 0.01)	**−0.27 (−0.42 to −0.11)**
	2	**−0.17 (−0.35 to 0.01)**	**−0.26 (−0.43 to −0.08)**	−0.11 (−0.28 to 0.07)	**−0.21 (−0.39 to −0.03)**
Free CML	1	**−0.16 (−0.33 to 0.00)**	**−0.31 (−0.47 to −0.15)**	−0.14 (−0.30 to 0.02)	**−0.19 (−0.35 to −0.02)**
	2	−0.18 (−0.45 to 0.10)	−0.24 (−0.51 to 0.02)	−0.12 (−0.39 to 0.15)	−0.07 (−0.34 to 0.20)
Free CEL	1	−0.14 (−0.33 to 0.04)	**−0.28 (−0.45 to −0.12)**	**−0.16 (−0.32 to 0.00)**	**−0.22 (−0.39 to −0.05)**
	2	−0.14 (−0.43 to 0.15)	−0.14 (−0.42 to 0.14)	−0.15 (−0.42 to 0.13)	−0.13 (−0.42 to 0.16)
Free MG-H1	1	**−0.21 (−0.37 to −0.04)**	**−0.25 (−0.42 to −0.09)**	−0.13 (−0.28 to 0.03)	**−0.21 (−0.37 to −0.05)**
	2	**−0.23 (−0.46 to 0.00)**	−0.12 (−0.34 to 0.09)	−0.06 (−0.28 to 0.15)	−0.13 (−0.35 to 0.10)
D-Lactate	1	−0.05 (−0.21 to 0.11)	0.00 (−0.16 to 0.17)	0.05 (−0.12 to 0.21)	−0.01 (−0.08 to 0.06)
	2	−0.04 (−0.20 to 0.13)	0.04 (−0.13 to 0.21)	0.08 (−0.10 to 0.25)	0.00 (−0.07 to 0.07)

All data were analyzed with linear regression. Bold values are significant. β values are presented per SD increase of Ln-normalized independent variable per SD increase of the outcome variables. All outcome variables were Ln transformed. Model 1 is adjusted for sex, age, and trial arm. Model 2 is further adjusted for BMI, presence of diabetes, smoking, eGFR, plasma cholesterol, and systolic blood pressure.

intra-arterial infusions of placebo or cells have influenced the associations with the prospective analyses. The glycation markers were measured before the interventions took place, and it is unlikely that they may have led to false-positive associations, because adjustment for trial arm did not influence the results. However, associations may have been weakened by introducing some random variation by the treatments, leading to an underestimation of the associations between the glycation markers and adverse outcomes. Furthermore, for our secondary analyses on mortality, in particular, we may have lacked statistical power, despite the fact that this relatively large cohort of SLI contained many adverse events.

The current study did not include a measurement of HbA_{1c} and we therefore could not fully investigate whether glycemic control influenced the results. However, this is unlikely, because we found no evidence that any of the associations differed for the presence of diabetes, and we found no obvious effect of adjustment for HbA_{1c} in previous studies investigating associations between plasma MGO levels and vascular outcomes (3,4).

This study has important implications, because the prognosis for patients with SLI remains poor, despite the rapid development of endovascular treatment options. Several compounds have been identified that quench formation of MGO, including pyridoxamine (21), carnosine (22), and small-molecule inducers of pyruvate kinase isozyme M2 (10). Modulation of the expression of Glo1 also remains of interest, because Glo1 can be boosted by a coformulation of *trans*-resveratrol and hesperetin, which has been shown to improve vascular function in overweight people (23), and overexpression of Glo1 in the BM of diabetic mice improved perfusion after hind limb artery ligation (24). Our findings suggest that modulation of the glyoxalase pathway may improve outcomes in SLI, because its major substrate, MGO, was associated with adverse outcomes and many indicators of poor outcome in SLI. Given the poor prognosis of SLI, the current study provides rationale for performing a study with MGO inhibitors and/or Glo1 inducers in individuals with SLI in order to achieve limb salvage and reduce mortality.

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