

Long-term effects of metformin on endothelial function in type 2 diabetes: a randomized controlled trial

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Abstract. de Jager J, Kooy A, Schalkwijk C, van der Kolk J, Lehert P, Bets D, Wulffelé MG, Donker AJ, Stehouwer CDA (Bethesda Diabetes Research Center, Hoogeveen; Academic Medical Center, Amsterdam; Bethesda Hospital, Hoogeveen; Cardiovascular Research Institute Maastricht, Maastricht University Medical Center, Maastricht; Clinical Laboratory, Bethesda Hospital, Hoogeveen, The Netherlands; FUCAM, Louvain Academy, Belgium; Clinical Research and Development, E. Merck, Amsterdam; Free University Medical Center, Amsterdam, The Netherlands). Long-term effects of metformin on endothelial function in type 2 diabetes: a randomized controlled trial. *J Intern Med* 2014; **275**: 59–70.

Objectives. We investigated whether metformin can improve endothelial function and decrease inflammatory activity, and thereby decrease the risk of atherothrombotic disease.

Subjects and design. A randomized, placebo-controlled trial with a follow-up period of 4.3 years set in the outpatient clinics of three nonacademic hospitals (Hoogeveen, Meppel and Coevorden Hospitals, the Netherlands). A total of 390 patients with type 2 diabetes treated with insulin were included. Either metformin 850 mg or placebo (one to three times daily) was added to insulin therapy. Urinary albumin excretion and plasma levels of von Willebrand factor (vWf), soluble vascular adhesion molecule-1 (sVCAM-1), soluble E-selectin (sE-selectin), tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), C-reactive protein (CRP) and soluble intercellular adhesion

molecule-1 (sICAM-1) were measured at baseline and after 4, 17, 30, 43 and 52 months.

Results. Metformin significantly reduced levels of vWF, sVCAM-1, t-PA, PAI-1, CRP and sICAM-1, which, except for CRP, remained significant after adjustment for baseline differences in age, sex, smoking and severity of previous cardiovascular (CV) disease. No effects on urinary albumin excretion or sE-selectin were observed. The improvements in vWf and sVCAM-1 statistically explained about 34% of the reduction in the risk of CV morbidity and mortality associated with metformin treatment in this study.

Conclusions. Metformin is associated with improvement in some (vWf and sVCAM-1) but not all markers of endothelial function, which may explain why it is associated with a decreased risk of CV disease in type 2 diabetes.

Keywords: cardiovascular disease outcome, endothelial function, HOME trial, metformin, type 2 diabetes.

Abbreviations: CI, confidence interval; CV, cardiovascular; DM2, type 2 diabetes; ELISA, enzyme-linked immunosorbent assay; HOME, Hyperinsulinemia: the Outcome of its Metabolic Effects; CRP, C-reactive protein; HR, hazard ratio; PAI-1, plasminogen activator inhibitor-1; sE-selectin, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular adhesion molecule-1; t-PA, tissue-type plasminogen activator; vWF, von Willebrand factor.

Introduction

Up to 75% of patients with type 2 diabetes (DM2) will die from cardiovascular (CV) complications [1], thus prevention of such complications in these patients is a crucial therapeutic target. Two key features of the pathophysiology of atherothrombosis are dysfunction of the endothelium and chronic low-grade inflammation of the vascular wall [2], which have both been shown in observational studies to be related to an increased risk of atherothrombotic disease [3].

In patients with DM2, metformin has been associated with less CV morbidity and mortality, at least in part independently of improvement in glycaemic control [4] and other risk factors, such as hypertension, obesity and dyslipidaemia [5]. These findings raise the question of whether metformin can improve endothelial function and decrease inflammatory activity and thereby decrease risk of atherothrombotic disease. There is some evidence from short-term studies that this may be the case [6–9], but no long-term, placebo-controlled data are available, and most short-term studies have focused only on markers of fibrinolysis [10–12], which may or may not reflect endothelial function [3].

In view of these considerations, we investigated the effects of metformin versus placebo on endothelial dysfunction and low-grade inflammation in a randomized controlled trial with follow-up for 4 years and 4 months in patients with insulin-treated DM2; the clinical outcomes of this study have been reported previously [5].

Subjects and methods

Trial design and study population

This study is part of the Hyperinsulinemia: the Outcome of its Metabolic Effects (HOME) trial to investigate the effects of metformin versus placebo on metabolism and vascular disease [5, 10]. Figure 1 shows the trial design and recruitment and retention of patients [5, 10, 11]. The HOME trial was conducted in the outpatient clinics of three nonacademic hospitals (Hoogeveen, Meppel and Coevorden Hospitals, the Netherlands). All participants provided written informed consent. The medical ethical committees of the three participating hospitals approved the trial protocol. The trial was conducted in accordance with Good Clinical Practice (CPMP/ICH/135/95; 1996) and with the Declaration of Helsinki (revised version, 2000).

Randomization, interventions and follow-up

Patients were randomly allocated to either placebo or metformin (in identical looking boxes) via a computer program. Either metformin 850 mg or placebo (one to three times daily) was added to insulin therapy.

Patients visited the clinics at the start of the prerandomization phase (3 months before randomization), at baseline (randomization to metformin or placebo), 1 month after baseline (to check the tolerance of the drug titration) and subsequently every 3 months until the end of the trial. During these visits a physical examination was carried out, a medical history was taken, and laboratory and urinary investigations were performed.

Laboratory investigations

Urinary albumin excretion was measured by immunoturbidimetry (Roche Diagnostics, Basel, Switzerland) at Meppel and Hoogeveen Hospitals and by nephelometry (BN ProSpec, Dade Behring, Marburg, Germany) at Coevorden Hospital [12]. Comparison of these methods according to Passing and Bablok [13, 14] showed no significant differences. Urinary albumin excretion was expressed as the albumin-to-creatinine ratio. Blood samples were collected at baseline and after 4, 17, 30, 43 and 52 months, and stored at -80°C until analysis. Plasma von Willebrand factor (vWf) antigen was measured using an in-house sandwich enzyme immunoassay, with rabbit anti-vWf antigen immunoglobulin G as the catching antibody and a peroxidase-conjugated rabbit anti-vWf antigen for detection (Dako, Copenhagen, Denmark). O-phenylenediamine (Sigma Chemical Co, St Louis, MO, USA) was used as substrate. Levels of vWf are expressed as percentage of antigen levels in normal pooled plasma (defined as 100%). The intra- and interassay coefficients of variation are 2.7% and 5.9%, respectively. Soluble vascular adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin (sE-selectin) were measured in duplicate using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Diacclone, Besançon, France). The intra- and interassay coefficients of variation are 1.1% and 3.1% for sVCAM-1, 1.8% and 4.2% for sICAM-1, and 2.7% and 5.9% for sE-selectin, respectively. The analysis of high-sensitivity C-reactive protein (CRP) was carried out using a Modular

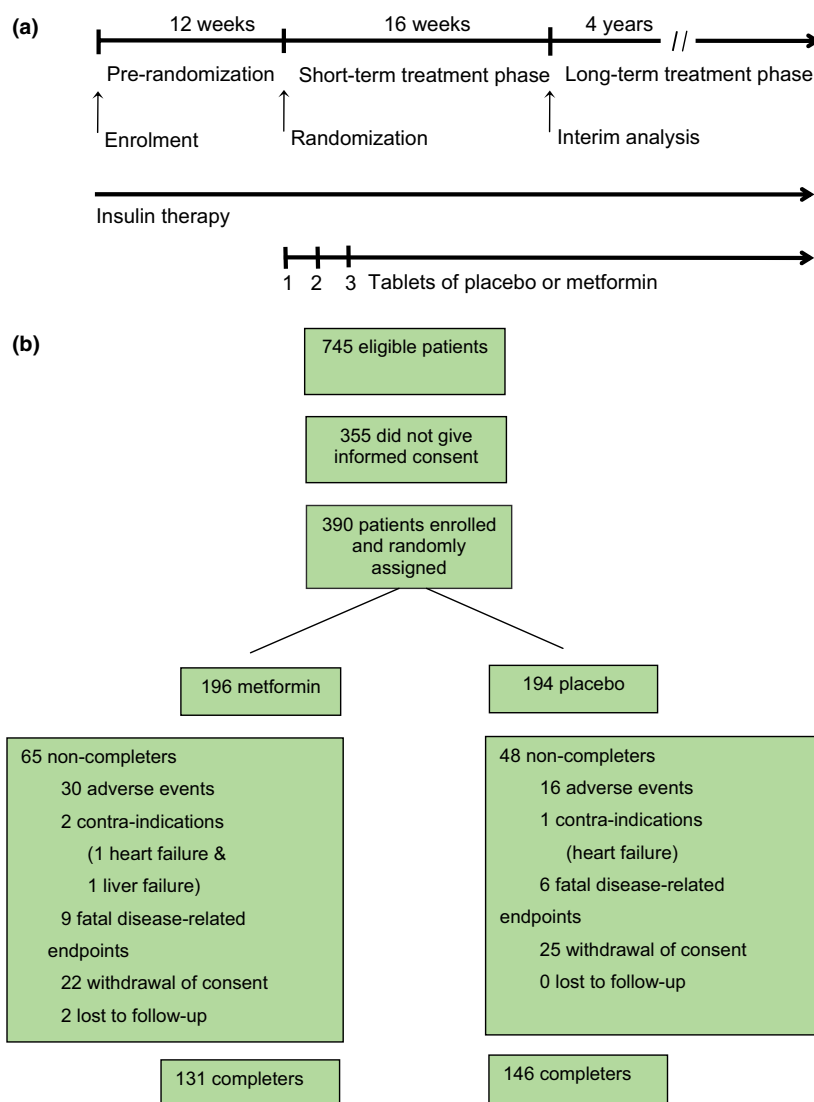


Fig. 1 Trial design (a) and flow diagram of recruitment and retention of patients in both groups (b).

Analytics P800 system with the Tina-quant[®] high-sensitivity CRP reagent kit (Roche Diagnostics), and anti-CRP antibodies coupled to latex microparticles to form an antigen–antibody complex. The intra- and interassay coefficients of variation are 0.4% and 2.7%, respectively. Tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) antigens were measured in duplicate using an ELISA reagent kit (Technoclone, Vienna, Austria). The intra- and interassay coefficients of variation are 2.6% and 7.9% for t-PA, and 3.8% and 6.8% for PAI-1, respectively.

In the HOME trial, markers of endothelial function and inflammation have been measured in samples

obtained at baseline and after 16 weeks of treatment, and the results previously reported [12]. To investigate the stability of the assay procedures, we compared previously obtained values with values obtained for the present investigation. Correlations between old and new measurements at baseline were 0.87 for urinary albumin excretion, 0.86 for vWf, 0.89 for sVCAM-1, 0.88 for sE-selectin, 0.92 for t-PA, 0.91 for PAI-1, 0.92 for CRP and 0.91 for sICAM-1.

We considered urinary albumin excretion and plasma levels of vWf, sVCAM-1, sE-selectin, sICAM-1, t-PA and PAI-1 to be markers of endothelial function [15–17], and plasma levels of CRP and

sICAM-1 to be markers of low-grade inflammation [18, 19]. Thus, sICAM-1 is a marker of both endothelial function and inflammation [19, 20]. Other laboratory methods have been described elsewhere [12].

Statistical analysis

The analyses include all patients who underwent randomization with available measurements at baseline (intention-to-treat sample using last observation carried forward; $n = 388$). Data with a skewed distribution were log-transformed before analysis. For the analyses of markers of endothelial function and inflammation, the end-point of interest was the percentage change in each variable from baseline, and the differences in these changes between the metformin and the placebo groups. The differences between the metformin and placebo groups were tested using the Student's *t*-test on log-transformed values. As log values are not directly interpretable, the antilog values are reported instead. In the case of log-transformed values, data are given as the geometric mean [95% confidence interval (CI)]. We used multivariate linear regression analysis to investigate whether metformin-associated improvements in markers of endothelial function and inflammation, if any, were independent of age, sex, smoking, changes in glycated haemoglobin (HbA1c), insulin dose, weight and the severity of previous CV disease computed as the sum score of CV disease events as follows: myocardial infarction absent = 0, present = 1; CV intervention (peripheral arterial reconstruction, percutaneous transluminal coronary angioplasty and coronary artery bypass graft) absent = 0, present = 1; transient ischaemic attack absent = 0, present = 1; stroke absent = 0, present = 1; dyspnoea New York Heart Association class absent = 0, I = 1, II = 2, III = 3, IV = 4; angina pectoris New York Heart Association absent = 0, I = 1, II = 2, III = 3, IV = 4; intermittent claudication, no = 0, more than 100 m = 1, 50 to 100 m = 2, less than 50 m = 3, rest = 4; and amputation absent = 0, present = 1 [5].

A *P*-value < 0.05 was considered statistically significant.

Multivariate mediation model of metformin treatment

Multivariate analyses were used to determine whether the effects of metformin to reduce CV outcome were mediated by the actions of metfor-

min on markers of endothelial function, inflammation, glycaemic control (HbA1c and fasting plasma glucose) and/or weight (waist-to-hip ratio and body mass index) [21]. As our sample size was not designed for such analyses, we compared various alternative models to provide appropriate statistical power for mediation tests. Poisson regression analysis of the total number of CV (TCV) events during the trial with adjustment for baseline age, gender and CV score was shown to provide a power of at least 0.75 when testing a maximum of three simultaneous mediators. We first assessed univariate mediating models for each mediator to eliminate any indirect effect of <5% of the overall direct effect of metformin. The *specific indirect effect* of metformin on TCV events via mediator M_i is defined as the product $a_i b_i$ of the two nonstandardized paths $a_i = \text{metformin} \rightarrow M_i$ and $b_i = M_i \rightarrow \text{TCV}$; the *total indirect effect* is the sum of the specific indirect effects; and the relative mediation effect was calculated as the ratio of the total indirect effect and the overall direct effect of metformin [22]. The best point and interval estimate of the total indirect effect were found by bootstrapping; we used both 'bias-corrected' and 'bias-corrected and accelerated' intervals [23].

Results

General trial results

Figure 1 shows the trial design and recruitment and retention of patients in both groups. A total of 390 subjects provided written informed consent and enrolled into the trial; 196 subjects were randomly assigned to receive metformin and 194 to receive placebo. Patients in the metformin group were slightly older than those receiving placebo (63.6 ± 9.6 vs. 59.1 ± 11.0 years), had a more extensive CV disease history (1.17 vs. 0.92) and were less often smokers. All other characteristics were comparable between the treatment groups (Table 1). Of the 390 included patients, 277 (71%) completed the HOME trial (Fig. 1). Those who did and did not complete the study did not differ with respect to duration of diabetes, previous occurrence or severity of CV disease, age or weight.

The mean dose in the metformin group was 2050 mg during the trial. There was no difference between the groups in the use of acetylsalicylic acid, antihypertensive drugs or statins. Metformin treatment was associated with less weight gain (mean difference, -3.07 kg; range, -3.85 to

Table 1 Baseline characteristics

	Placebo (n = 194)	Metformin (n = 196)
Demographic characteristics ^a		
Men/women	97/97	81/115
Age (years) ^a	59 (11)	64 (10)
Current smoking n (%)	59 (30)	38 (19)
Duration of diabetes (years) ^a	12 (8)	14 (9)
Insulin treatment (years)	6 (6)	7 (8)
Concomitant medication		
Acetylsalicylic acid n (%)	82 (42)	78 (40)
Lipid-lowering drugs n (%)	31 (16)	31 (16)
Blood pressure-lowering drugs n (%)	76 (39)	92 (47)
ACE inhibitors n (%)	31 (16)	35 (18)
Metabolic variables		
Weight (kg)	87 (15)	85 (16)
Body mass index (kg m ⁻²)	30 (5)	30 (5)
Waist-to-hip ratio		
Men	1.03 (0.1)	1.02 (0.1)
Women	0.93 (0.1)	0.92 (0.1)
Plasma HbA1c (%)	7.9 (1.2)	7.9 (1.2)
Preprandial glucose (mmol L ⁻¹)	8.8 (1.8)	8.6 (1.8)
Postprandial glucose (mmol L ⁻¹)	10.2 (2.0)	10.2 (2.1)
Plasma insulin (pmol L ⁻¹)	301 (686)	248 (545)
Daily dose of insulin (IU day ⁻¹)	64 (25)	62 (29)
Systolic blood pressure (mmHg)	159 (25)	160 (25)
Diastolic blood pressure (mmHg)	86 (11)	86 (12)
Total cholesterol (mmol L ⁻¹)	5.5 (1.2)	5.6 (1.3)
LDL cholesterol (mmol L ⁻¹)	3.4 (1.0)	3.6 (1.1)
Triglycerides (mmol L ⁻¹)	1.9 (1.5)	1.7 (1.2)
HDL cholesterol (mmol L ⁻¹)	1.3 (0.4)	1.3 (0.4)
Diabetic complications		
Cardiovascular n (%)	53 (29)	59 (30)
Amputation n (%)	3 (2)	4 (2)
Paraesthesias n (%)	79 (41)	83 (42)
Cardiovascular disease severity score	0.92 (1.3)	1.17 (1.4)

ACE, angiotensin-converting enzyme; HbA1c, glycated haemoglobin.

Data are mean (SD), unless otherwise indicated.

^aDifferences at baseline between the groups; all end-point results were adjusted for these differences.

−2.28 kg; $P < 0.001$), better glycaemic control (mean difference in HbA1c, −0.4%; 95% CI, −0.55 to −0.25; $P < 0.001$), despite the aim of similar glycaemic control in both groups, and reduced insulin requirements (mean reduction, 19.63 IU day⁻¹; 95% CI, 24.91–14.36 IU day⁻¹; $P < 0.001$). Metformin treatment was not associ-

ated with a decrease in blood pressure or improvement in lipid profile (data not shown).

Markers of endothelial function and inflammation

Compared with placebo (Table 2 and Fig. 2), metformin treatment was not associated with a change

Table 2 Markers of endothelial function and inflammation at baseline and after 4.3 years (metformin compared to placebo)

	Baseline (t_0)		Last visit (t_f)		Change (%)		P	
	Placebo (P)	Metformin (M)	Placebo (P)	Metformin (M)	P t_1 vs. t_0	M t_1 vs. t_0	M vs. P	M vs. P
Markers of endothelial function								
UAE (mg/mmol)	1.03 (0.79 to 1.35)	1.00 (0.76–1.32)	0.83 (0.64–1.07)	0.84 (0.64–1.10)	–16 (–33 to +5)	–3 (–26 to +27)	+16 (–19 to +64)	0.422
vWf (%)	118 (112–125)	123 (117–130)	132 (125–140)	123 (116–130)	+12 (+7 to +17)	–1 (–4 to +3)	–11 (–16 to –6)	<0.001
sVCAM-1 (ng/mL)	744 (718–771)	766 (740–794)	779 (748–810)	762 (734–791)	+5 (+3 to +7)	–1 (–3 to +1)	–5 (–8 to –3)	<0.001
sE-selectin (ng/mL)	84.8 (79.2–90.7)	87.1 (82.0–92.5)	81.8 (76.3–87.7)	82.4 (77.1–88.0)	–3 (–7 to 0)	–5 (–8 to –2)	–2 (–7 to +3)	0.449
t-PA (ng/mL)	6.7 (6.3–7.2)	6.9 (6.5–7.4)	7.1 (6.7–7.6)	6.21 (5.8–6.7)	+6 (+1 to +11)	–10 (–14 to –6)	–15 (–20 to –9)	<0.001
PAI-1 (ng/mL)	45.8 (39.8–52.7)	39.7 (34.3–46.1)	48.7 (42.0–56.4)	33.6 (28.7–39.4)	+7 (–4 to +19)	–15 (–23 to –7)	–21 (–31 to –9)	0.001
Markers of inflammation								
CRP (mg L ^{–1})	3.06 (2.61–3.58)	3.06 (2.63–3.56)	3.12 (2.67–3.66)	2.61 (2.24–3.05)	+7 (–6 to +21)	–12 (–22 to 0)	–17 (–31 to –1)	0.036
sICAM-1 (ng mL ^{–1})	488 (470–507)	491 (472–511)	506 (486–527)	484 (463–506)	+4 (+1 to +6)	–1 (–4 to +1)	–5 (–8 to –2)	0.004

CRP, C-reactive protein; PAI-1, plasminogen activator inhibitor-1; sE-selectin, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; t-PA, tissue-type plasminogen activator; UAE, urinary albumin excretion; vWf, von Willebrand factor; sVCAM-1. Data at baseline and follow-up are mean with 95% confidence interval (CI) or geometric mean with 95% CI (when log-transformed). Change is expressed as the mean percentage change with 95% CI.

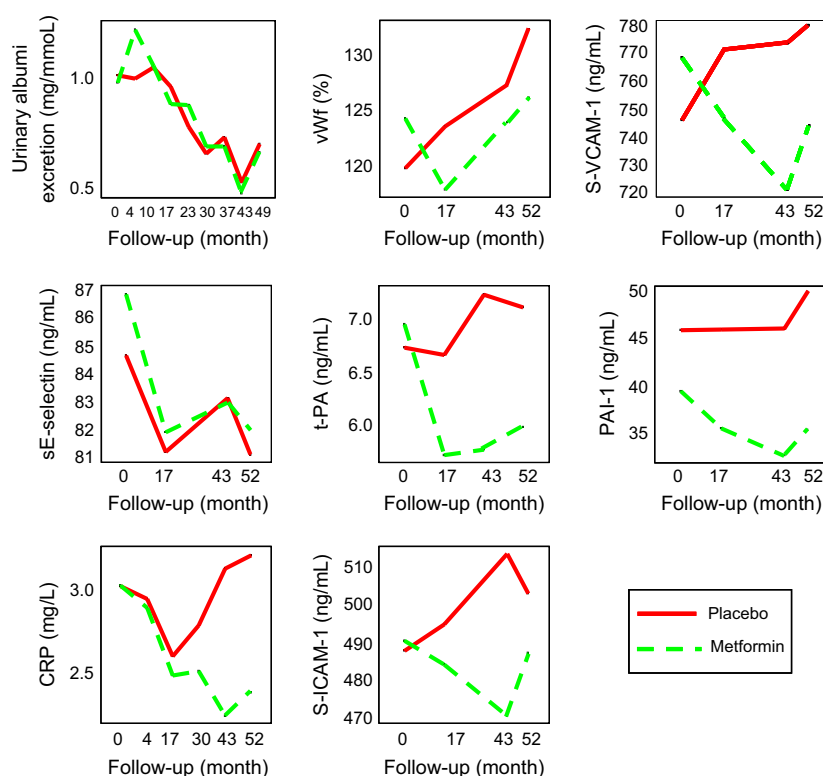


Fig. 2 Changes in markers of endothelial function and inflammation during the follow-up period in the metformin and placebo groups.

in urinary albumin excretion (16%; -19 to $+64$; $P = 0.422$). However, metformin treatment was associated with the following changes: a decrease in vWf of 11% (-16 to -6 ; $P < 0.001$); a decrease in sVCAM-1 of 5% (-8 to -3 ; $P < 0.001$); no significant change in sE-selectin (2%; -6 to $+3$; $P = 0.45$); a decrease in t-PA of 15% (-20 to -9 ; $P < 0.001$); a decrease in PAI-1 of 21% (-31 to -9 ; $P = 0.001$); a decrease in CRP of 17% (-31 to -1 ; $P = 0.036$); and a decrease in sICAM-1 of 5% (-8 to -2 ; $P = 0.004$).

Adjustments for age, sex, smoking and severity of previous CV disease did not essentially change these results, except for CRP (-28% ; -94 to $+39$; $P = 0.41$). The effect on sICAM-1 did not change substantially, but was no longer statistically significant (decrease of 5%; -10 to 0 ; $P = 0.06$). Adjustment for changes during the trial in HbA1c, insulin dose and weight, in addition to the baseline variables above, also did not affect the results, except that, after adjustment for the change in HbA1c and insulin dose, the effect of metformin on PAI-1 and sICAM-1 was no longer significant: -23% (-50 to $+3$; $P = 0.08$) and $+2\%$ (-3 to $+7$; $P = 0.52$), respectively. On the other hand, after

adjustment for the change in HbA1c and insulin dose, the effect of metformin on sE-selectin became significant: $+9\%$ ($+2$ to $+15$; $P = 0.01$).

Is the effect of metformin on CV disease mediated by improvement in endothelial function?

We assessed separate univariate mediating models for each potential mediator to select those with a minimum relative mediation effect ($>5\%$ of the direct effect). For each mediator, we evaluated both the last value and the change in time from baseline until final value. Only the changes in vWf and sVCAM-1 (both log-transformed values) were selected; weight, lipids, HbA1c, fasting plasma glucose and insulin dose were not selected. Thus, the main multivariate mediation model consisted of a Poisson regression on TCV events predicted by vWf and sVCAM-1 adjusted for baseline CV score, age and gender. The indirect effect of metformin on TCV events through the mediating effect of the decrease in vWf over time was estimated as a rate ratio of 0.93 (95% CI, 0.84 to 1.01, $P = 0.059$); thus, the effect of reducing TCV events was $100(1-0.93) = 7\%$, compared with

placebo. The indirect effect of metformin on TCV events through the mediating effect of the decrease in sVCAM-1 over time was estimated as a rate ratio of 0.95 (95% CI, 0.85 to 1.04, $P = 0.181$); thus, the decrease in TCV events was 100 $(1 - 0.95) = 5\%$, compared with placebo. The rate ratio of the total mediating effect of vWF and sVCAM-1 was 0.88 (95% CI, 0.78–0.99; $P = 0.028$), and the relative mediation effect was estimated as a rate ratio of 0.34 (95% CI, 0.09–0.68). Statistical contrasts between vWF and sVCAM-1 did not allow rejection of the equality of the two effects ($P = 0.47$). Using time to first CV event and a proportional hazards technique, the results were similar but did not reach statistical significance (total mediating effect, $P = 0.12$).

Discussion

There were four main findings in the present study of the long-term effects of metformin on endothelial function and inflammation in patients with DM2 treated with insulin. First, metformin significantly reduced levels of vWF, sVCAM-1, t-PA, PAI-1 and sICAM-1. Of note, our results show that this is not a temporary phenomenon, but persists over time. Secondly, the favourable changes in vWF, sVCAM-1, t-PA and PAI-1 appeared to be partly independent of metformin-associated changes in glycaemic control, insulin dose and weight, whereas the reduction in sICAM-1 was entirely mediated through metformin-induced improvements in glycaemic control and lower insulin levels. Thirdly, after adjustment for baseline imbalances in age, sex, smoking and previous CV disease, metformin did not reduce CRP levels. Fourthly, metformin-associated changes in endothelial dysfunction, as estimated by vWF and sVCAM-1, explained about 34% of the reduction in the risk of CV morbidity and mortality associated with metformin. Taken together, these results suggest that, in DM2, metformin improves endothelial function and decreases the risk of CV disease to some extent (about one-third) through improving endothelial dysfunction.

In addition, we evaluated other potential mechanisms through which metformin may reduce CV outcome. However, we did not find any evidence for a role of weight, lipids, HbA1c, fasting plasma glucose or insulin dose. In particular, although the metformin-dependent prevention of weight gain during the study may be relevant, it did not contribute to the reduction in CV outcome within

the study period. This is in contrast to our previously reported analyses [5]; however, in the earlier analyses, we did not take into account the important effects of baseline weight and CV disease history, which in the current analyses confounded the influence of the changes in weight during the trial on CV outcomes. Another potentially important issue is the 'obesity paradox': as lowering weight may be the result of CV disease itself (cachexia) and its treatments (e.g. dietary advice and compliance, or diuretic therapy and fluid restriction), weight loss (or less weight gain) may be an indicator of disease severity and thus be *positively* associated with CV disease risk. In sum, weight change during the trial is subject to a number of influences that we could not take into account, and this may explain why we did not find that less weight gain was favourably associated with CV outcome.

DM2 is a state of generalized endothelial dysfunction, with impairment of many endothelial functions, such as regulation of vasomotor tone, leucocyte adhesion, haemostasis and fibrinolysis, in many vascular beds [15]. We have previously found that endothelial dysfunction in DM2 is progressive over time [24], is strongly associated with CV disease risk [5] and can explain ~34% of the excess CV mortality associated with DM2 [24]. Against this background, in the current study, we found that metformin treatment was associated with decreases in the plasma levels of vWf, sVCAM-1, t-PA and PAI-1, that is, with improvement in the endothelial regulation of haemostasis (vWf), leucocyte adhesion (sVCAM-1 and possibly sICAM-1) and fibrinolysis (t-PA and PAI-1). For vWf, t-PA and PAI-1, these findings are in accordance with previous experience in individuals with diabetes and without diabetes [7–9, 25]. To the best of our knowledge, no placebo-controlled data on the effect of metformin on sVCAM-1 in patients with diabetes exist, whereas actively controlled data on sVCAM-1 in patients with or without diabetes have so far yielded contradictory results [25–28]. It is noteworthy that changes in the plasma levels of vWf, sVCAM-1, t-PA and PAI-1 were partly (~60–70%) independent of metformin-associated favourable changes in body weight, glycaemic control and insulin dose. Taken together, these findings raise the possibility that improvement of endothelial function by metformin may represent a partly glucose-independent pathway through which metformin decreases risk of CV disease in DM2 [4].

An important assumption in this reasoning is that plasma levels of these markers are valid indicators of endothelial function. This, in turn, requires that endothelial cells are the major source of the plasma concentration of these proteins and that protein concentration is determined by synthesis rather than by clearance. However, the validity of these assumptions is uncertain [15]. Of the markers investigated, only sE-selectin and t-PA are synthesized exclusively by endothelial cells. t-PA in plasma binds to PAI-1, and t-PA concentrations may mainly reflect the concentration of PAI-1, which is synthesized not only by endothelial cells, but also by hepatocytes and adipocytes. In addition, there is almost no information on the regulation of the clearance of these proteins in DM2, except for vWf, for which there is indirect evidence that its plasma concentration in DM2 is determined by synthesis rather than clearance [29].

Urinary albumin excretion tended to increase during short-term treatment with metformin in this study; although this is consistent with our previous observations, it had been considered to be a chance finding [12]. The current long-term results seem to confirm this explanation of the unexpected short-term findings, as no effect of metformin on urinary albumin excretion was found after 4.3 years. Previous studies of the effect of metformin on urinary albumin excretion showed either no effect or a decrease [7, 30]. The interpretation of the finding that metformin improves endothelial dysfunction, but not urinary albumin excretion, is unclear. Urinary albumin excretion depends on glomerular albumin permeation (itself dependent on pressure, permeability and surface area) and tubular reabsorption. Microalbuminuria has been strongly associated with endothelial dysfunction and is postulated to reflect increased endothelial permeability to macromolecules [31]. The simplest explanation for our findings, therefore, is that metformin did not improve endothelial permeability even if it did improve other endothelial functions.

We found no long-term effect of treatment with metformin on sE-selectin. Unexpectedly, after adjustments for changes in HbA1c and insulin dose, treatment with metformin was associated with an increase in sE-selectin levels, suggesting that metformin may have an intrinsic effect on sE-selectin, which is obscured by the lowering effect on sE-selectin of metformin-induced improve-

ments in glycaemic control. Most previous studies showed either no significant reductions in sE-selectin, or decreases that were attributable to changes in lipid profile or glycaemic control [26–28]. In *in vitro* studies in human endothelial cells, metformin dose-dependently inhibited tumour necrosis factor- α (TNF- α)-induced NF- κ B-dependent gene expression of E-selectin, possibly through AMP-activated protein kinase activation [32, 33]. The metformin-associated increase in plasma sE-selectin levels is therefore difficult to explain and may theoretically be related to increased shedding from the cell membrane, or to decreased clearance of plasma sE-selectin. Although these possibilities require further investigation, it is important to note that we did not find any evidence that the metformin-associated increase in plasma sE-selectin affected the risk of CV events.

Metformin did not reduce levels of CRP after adjustment for the baseline variables age, smoking and previous CV disease severity. Metformin did reduce sICAM-1 levels, which was explained by metformin-associated improvements in glycaemic control and insulin levels. Studies of sICAM-1 and CRP, in patients with and without DM2, have so far yielded contradictory results [25, 27, 28]. Taken together, these data do not support the concept that metformin decreases inflammatory activity independently of improvement of glycaemic control, although it must be emphasized that this conclusion is limited by the fact that we measured only two markers of inflammation. On the other hand, other markers of inflammation, such as IL-6 and TNF- α , are generally quite highly correlated with CRP [34].

Epidemiological data have indicated that endothelial dysfunction can explain about 34% of the increased CV mortality associated with DM2 [24]. Metformin has been associated with lower CV morbidity and mortality [4, 5]. In our study, metformin improved endothelial dysfunction and these changes in endothelial function, specifically in vWF and sVCAM-1, explained ~34% of the reduced risk of CV morbidity and mortality associated with metformin; it is remarkable that this estimate is the same as that obtained in observational analyses [24].

We showed that the effects of metformin on endothelial function were partly unrelated to decreases in hyperglycaemia, insulin dose and weight,

suggesting that metformin may have some direct effects on the endothelium. Alternatively or additionally, metformin may improve endothelial function by decreasing levels of advanced glycation end products [35–37], by altering the secretion of adipocyte-derived mediators (such as free fatty acids, leptin, resistin and adiponectin) [38–40], by decreasing inflammatory activity in ways not reflected by CRP and sICAM-1 [41, 42] and/or by improving insulin sensitivity, of which a change in insulin dose may be an insufficiently accurate marker. These possibilities require further study.

Strengths of the present study include its randomized, placebo-controlled, double-blind design, the relatively long follow-up of 4.3 years with frequent serum collection, and its nonacademic setting and therefore value in a community setting. This study had limitations that also need to be considered. First, there was an imbalance between the two treatment groups after randomization. Although we adjusted for this in all analyses, we cannot rule out some residual confounding. Secondly, the results regarding low-grade inflammation must be interpreted with caution. Low-grade inflammation was estimated by two markers, CRP and sICAM-1, whereas endothelial dysfunction was represented by six markers. This may have led to an underestimation of the effect of metformin on low-grade inflammation.

In conclusion, in patients with DM2 treated with insulin, 4.3 years of metformin treatment, compared to placebo, was associated with improvements in plasma markers of vWF, sVCAM-1, t-PA, PAI-1 and sICAM-1, all of which (except the changes in sICAM-1) were partly independent of changes in HbA1c, insulin dose and weight. Metformin may thus have specific effects on endothelial function, which may explain why it appears to be associated with a decreased risk of CV disease in DM2. Improvements in endothelial dysfunction (i.e. changes in vWf and sVCAM-1) explained about 34% of the reduced risk of macrovascular morbidity and mortality associated with the use of metformin.

Author contributions and disclosures

A. Kooy, A. J. Donker and C. D. A. Stehouwer designed the study; J. de Jager, M. G. Wulffélé, D. Bets, J. van der Kolk, C. Schalkwijk and A. Kooy collected the data; J. de Jager, A. Kooy, P. Lehert and C. D. A. Stehouwer analysed and interpreted the data; J. de Jager, A. Kooy and C. D. A.

Stehouwer drafted the manuscript; A. Kooy, P. Lehert, C. Schalkwijk, A. B. Donker and C. D. A. Stehouwer revised the manuscript for important intellectual content; J. de Jager, P. Lehert, A. Kooy and C. D. A. Stehouwer were involved in statistical analysis; A. Kooy obtained funding; J. de Jager, M. G. Wulffélé, A. Kooy, D. Bets, J. van der Kolk and C. Schalkwijk provided administrative and technical support; A. Kooy, P. Lehert and C. D. A. Stehouwer supervised the study.

Sources of funding

This study, as part of the HOME trial, was supported by grants from Altana, Lifescan, Merck Serono, Merck Sharp & Dohme and Novo Nordisk. The sponsors had no role in the design and conduct of the study; the collection, analysis and interpretation of the data; or the preparation, review or approval of the manuscript.

Conflict of interest

P. Lehert is a consultant for Merck Serono. Other authors have no conflict of interest connected to this paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Multivariate mediation model of metformin treatment: statistical development. ■