

Circulating Dickkopf-1 in Diabetes Mellitus: Association With Platelet Activation and Effects of Improved Metabolic Control and Low-Dose Aspirin

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Background—Dickkopf-1 (DKK-1) is a major regulator of the Wnt signaling pathway, involved in inflammation, atherogenesis, and the regulation of glucose metabolism. Because platelets are major contributors to circulating levels of DKK-1 in other clinical settings, we aimed at characterizing the platelet contribution to DKK-1 in type 2 diabetes mellitus (T2DM) and evaluating associations of DKK-1 with glucose metabolism, platelet activation, and endothelial dysfunction.

Methods and Results—A cross-sectional comparison of DKK-1, soluble CD40L (sCD40L; reflecting platelet-mediated inflammation), asymmetric dimethylarginine (ADMA; marker of endothelial dysfunction), and urinary 11-dehydro-thromboxane B₂ (in vivo marker of platelet activation) was performed among 214 diabetic patients (90 receiving aspirin at 100 mg/day) and 30 healthy controls. Plasma DKK-1 levels were markedly higher in patients with T2DM than in healthy patients ($P<0.0001$). DKK-1 levels were significantly lower in diabetic patients receiving compared with those not on aspirin treatment ($P=0.008$); in the latter, DKK-1 was significantly correlated with 11-dehydro-thromboxane B₂, ADMA, and CD40L ($\rho=0.303$, $P<0.0001$, $\rho=0.45$, $P<0.0001$, and $\rho=0.37$, $P<0.0001$, respectively) but not with glycemic control or DM duration. Among patients not receiving aspirin, improvement of metabolic control in a subgroup of newly diagnosed patients treated with acarbose for 20 weeks and in a group treated with rosiglitazone for 24 weeks was associated with concurrent significant reductions in DKK-1 ($P=0.005$ and $P=0.004$) and 11-dehydro-thromboxane B₂ ($P=0.005$ and $P=0.004$).

Conclusions—Circulating DKK-1 is increased in T2DM and associated with endothelial dysfunction and platelet activation. Plasma DKK-1 levels are reduced with improvement of glycemic control and low-dose aspirin treatment. (*J Am Heart Assoc.* 2014;3:e001000 doi: 10.1161/JAHA.114.001000)

Key Words: diabetes mellitus • DKK-1 • endothelial dysfunction • inflammation • platelet activation • platelet-derived factor

Diabetes mellitus (DM) is associated with accelerated atherogenesis, resulting in premature ischemic manifestations of coronary, cerebrovascular, and peripheral arterial disease, and enhanced platelet activation seems to be involved in this accelerated atherosclerotic process. Abnormalities in ex vivo platelet function in patients with DM have

been described by several groups, and chronic hyperglycemia is a major contributor to in vivo platelet activation in type 2 DM (T2DM), suggesting a direct link between the degree of glycemic control and platelet activation in these patients.^{1,2} The role of platelets in atherothrombosis seems not only to be related to their pro-thrombotic properties but also to involve their role as inflammatory cells. On activation, they release and express inflammatory mediators such as CD40 ligand (CD40L),³ the fractalkine/CX3CL1 receptor CX3CR1,⁴ and migration inhibitory factor-related protein (MRP)8/14,⁵ inducing an inflammatory response in adjacent leukocytes and endothelial cells. We have previously provided evidence for enhanced release of soluble (s)CD40L by platelets from T2DM patients, involving thromboxane (TX)-dependent mechanisms.⁶

The wingless (Wnt) proteins are a group of highly conserved secreted mediators that regulate a wide range of cellular processes such as proliferation, survival, cell fate determination, and migration.^{7–10} More recently, Wnt

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signaling was shown to modulate several processes with relevance to vascular diseases and atherosclerosis such as endothelial function, vascular smooth muscle cell (SMC) proliferation, angiogenesis, and inflammation, mediating both inflammatory and anti-inflammatory effects. Several studies have also suggested the possible involvement of Wnt signaling in the regulation of glucose metabolism.¹¹

The Wnt pathways are regulated by multiple families of secreted antagonists or modulators including dickkopfs (DKK). We and others have previously shown increased expression of DKK-1 both systemically and within the atherosclerotic lesion in patients with atherosclerotic disorders,^{12,13} potentially involved in the inflammatory interaction between platelets and endothelial cells.⁴ Based on the high levels of DKK-1 in platelets as demonstrated by us and others^{14,15} and the enhanced platelet activation in vivo in T2DM, we hypothesized that these patients would display increased circulating DKK-1. Furthermore, we tested the hypothesis that increased concentrations of DKK-1 observed in T2DM may derive, at least in part, from TX-dependent platelet activation. We also examined whether the formation of DKK-1 was related to the extent of glycemic control and endothelial dysfunction in this setting as well as whether improved metabolic control and low-dose aspirin (ASA) modulated these processes.

Materials and Methods

Subjects

Two hundred fourteen patients (97 women, 117 men; mean age 64 ± 9 years) with T2DM, as defined in accordance with the criteria of the American Diabetes Association,¹⁶ and 30 healthy subjects (20 women, 10 men; mean age 45 ± 14 years) were enrolled in the study. The baseline characteristics of diabetic patients are reported in the Table. Exclusion criteria were (1) clinically significant hepatic, renal, cardiac, or pulmonary insufficiency; (2) history of malignant neoplasms (diagnosed and treated within the last 5 years); (3) autoimmune disorders and type 1 DM; (4) a recent history (<6 months) of thrombotic events, pregnancy, or lactation; (5) regular use of estroprogestin, iron, antioxidants, non-steroid anti-inflammatory drugs, or antiplatelet agents other than ASA.

At the time of the study, diabetic patients were being treated with diet alone, insulin alone, diet plus an oral hypoglycemic agent, or an oral hypoglycemic agent plus insulin. Of the diabetic patients, 90 were also being treated with low-dose ASA (100 mg/day) for primary or secondary cardiovascular prevention.¹⁷

Diabetic patients with arterial hypertension or hypercholesterolemia were included if well controlled with stable drug

therapy: 130 (60.7%) had arterial hypertension (64 receiving ASA treatment), defined as current systolic/diastolic blood pressure $>130/85$ mm Hg; 153 (71.5%) were hypercholesterolemic (73 receiving ASA treatment), in accordance with the ATP III criteria.¹⁸

Diabetic patients were examined for the presence of microvascular and macrovascular complications. Of the 214 patients, 29 (13.5%) (19 receiving ASA treatment) had microvascular complications (nephropathy, detected by the presence of persistent microalbuminuria between 30 and 300 mg/24 hr, in at least 2 of 3 consecutive 24-hour collections, with a glomerular filtration rate ≥ 90 mL/min per 1.73 m², as calculated with the Modification of Diet in Renal Disease formula; retinopathy, based on fundus oculi examination), and 51 (23.8%) (46 receiving ASA treatment) had a history or physical examination positive for evidence of macrovascular complications (cerebrovascular disease, coronary heart disease, or peripheral arterial disease). Written informed consent was obtained from each subject participating in the study. The local ethics committee approved the protocol.

Design of the Studies

First, a cross-sectional comparison of circulating DKK-1, soluble CD40L (sCD40L) as a marker of platelet-mediated inflammation, asymmetric dimethylarginine (ADMA), as a marker of endothelial dysfunction, and urinary 11-dehydro-TXB₂ was performed among all patients and controls. All subjects were studied as outpatients, after a 12-hour fast, and had an overnight urine collection performed immediately before blood sampling. Urine samples were added with the antioxidant 4-hydroxy-Tempo (1 mmol/L) (Sigma Chemical Co) and stored at -20°C until extraction. Blood samples were obtained for lipid levels as well as routine blood chemistry to check inclusion and exclusion criteria. Second, to test the hypothesis of a platelet origin of DKK-1, 90 T2DM patients receiving ASA (100 mg/day) were compared with the 124 patients not receiving ASA treatment. Finally, to test the hypothesis that DKK-1 release in the circulation is an early event in the natural history of DM, possibly reversible with amelioration of metabolic control, we evaluated the separate effects of 2 different interventions modulating metabolic control on the biochemical variables under study. Specifically, we evaluated the effects of acarbose, an α -glucosidase inhibitor counteracting hyperglycemia and insulin resistance, known to prevent or delay the occurrence of DM,¹⁹ on plasma DKK-1 concentrations within a multicenter, randomized, double-blind, placebo-controlled study²⁰ in a subgroup of 20 diabetic patients from our diabetes center for whom plasma samples were still available. In this protocol, patients with T2DM in an early stage, as defined by a known disease

Table. Baseline Characteristics of the Type 2 Diabetic Patients

Variables	Patients With Type 2 Diabetes Mellitus			
	All (N=214)	Not Receiving ASA (n=124)	Receiving ASA (n=90)	P Value*
Male sex, n (%)	117	59 (47.6)	58 (64.4)	0.018
Age (y), median (IQR)	64 (59 to 70)	63 (56 to 70)	66.9 (61.9 to 69.7)	0.007
BMI, kg/m ²	28.7 (25.6 to 31.2)	29.4 (26.4 to 32)	27.9 (24.6 to 31.1)	0.027
Diabetes duration, y	5 (1 to 12)	3 (1 to 8)	8 (3 to 18)	<0.0001
Smoking	7 (3.3)	4 (3.3)	3 (3.6)	0.597
Diabetes duration >1 y, n (%)	143 (66.8)	70 (56.4)	73 (81.1)	<0.0001
Systolic blood pressure, mm Hg	135 (130 to 142.9)	131.5 (120 to 145)	135 (130 to 140.4)	0.377
Diastolic blood pressure, mm Hg	80 (70 to 88)	80 (70 to 90)	80 (70 to 84.5)	0.240
Fasting plasma glucose, mmol/L	7.22 (6.18 to 8.61)	7.42 (6.22 to 8.82)	7.06 (6.17 to 8.32)	0.176
Hemoglobin A _{1c} , mg/dL	6.9 (6.4 to 7.6)	6.9 (6.5 to 7.6)	6.8 (6.3 to 7.7)	0.730
Hypertension, n (%)	130 (60.7)	66 (53.2)	64 (71.1)	0.014
Hypercholesterolemia, n (%)	153 (71.5)	80 (64.5)	73 (81.1)	0.004
Total cholesterol, mmol/L	4.9 (4.31 to 5.56)	4.94 (4.39 to 5.66)	4.75 (4.06 to 5.43)	0.147
HDL cholesterol, mmol/L	1.24 (1.06 to 1.47)	1.24 (1.06 to 1.47)	1.24 (1.02 to 1.5)	0.657
Triglycerides, mmol/L	1.36 (0.97 to 1.95)	1.48 (1.04 to 2.16)	1.31 (0.88 to 1.87)	0.047
LDL cholesterol, mmol/L	2.82 (2.3 to 3.36)	2.87 (2.4 to 3.3)	2.76 (2.16 to 3.46)	0.469
Creatinine, μmol/L	76.6 (61.8 to 88.4)	64.5 (54.8 to 80.4)	84.8 (71.6 to 97.2)	<0.0001
Microvascular complications, n (%)	29 (13.5)	10 (8.1)	19 (21.1)	0.003
Macrovascular complications, n (%)	51 (23.8)	5 (4.0)	46 (51.1)	<0.0001
Previous MI, n (%)	16 (7.5)	3 (2.4)	13 (14.4)	<0.0001
Previous stroke, n (%)	7 (3.3)	1 (0.8)	6 (6.7)	0.012
Previous TIA, n (%)	7 (3.3)	0 (0)	7 (7.8)	0.001
Carotid stenosis, n (%)	23 (10.7)	7 (5.6)	16 (17.8)	0.013
Medical treatment				
Statins, n (%)	80 (37.4)	34 (27.4)	46 (51.1)	0.001
Metformin, n (%)	116 (54.2)	59 (47.6)	57 (63.3)	0.088
PPAR-γ, n (%)	19 (8.8)	5 (4.0)	14 (15.5)	0.08
Sulfonylureas, n (%)	47 (22)	17 (13.7)	30 (33.3)	0.002
Insulin, n (%)	22 (10.3)	7 (5.6)	15 (16.7)	0.022
Glinides, n (%)	12 (5.6)	2 (1.6)	10 (11.1)	0.006
Incretins, n (%)	3 (1.4)	3 (2.4)	0	0.257
Ezetimibe, n (%)	1 (0.5)	1 (0.8)	0	1.000
Fibrates n (%)	5 (2.3)	1 (0.8)	4 (4.4)	0.193
PUFA, n (%)	8 (3.7)	2 (1.6)	6 (6.7)	0.079
ACE-inhibitors, n (%)	61 (28.5)	32 (25.8)	29 (32.2)	0.442
ARBs, n (%)	38 (17.7)	14 (11.3)	24 (26.6)	0.006
Diuretics, n (%)	43 (20.1)	17 (13.7)	26 (28.9)	0.010
β-blockers, n (%)	30 (14)	10 (8.1)	20 (22.2)	0.005
CCA, n (%)	30 (14)	14 (11.3)	16 (17.8)	0.233
PPI, n (%)	27 (12.6)	8 (6.4)	19 (21.1)	0.020

ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; ASA indicates aspirin; BMI, body mass index; CCA, calcium channel antagonist; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; MI, myocardial infarction; PPAR-γ, peroxisome proliferator-activated receptor-γ; PPI, proton-pump inhibitor; PUFA, polyunsaturated fatty acids; TIA, transient ischemic attack.

*By Mann-Whitney, χ^2 , or Fisher exact test, as appropriate.

duration <6 months, presenting at the time of recruitment with hemoglobin (Hb)_{A1c} values <7% in the absence of any treatment affecting glycemic control, were randomly assigned to receive acarbose (up to 100 mg 3 times daily) or placebo, in addition to dietary counseling, for 20 weeks. Finally, we also evaluated the effects of rosiglitazone, a peroxisome proliferator-activated receptor- γ (PPAR γ) agonist, on these parameters, in 20 patients with T2DM for <10 years, HbA_{1c}<9.5%, already treated with metformin 2000 mg/day, not receiving insulin or insulin secretagogue or thiazolidinedione therapy. They were randomly assigned to receive rosiglitazone (8 mg/day), or placebo for 24 weeks. Subjects took 6 tablets daily (rosiglitazone 2 mg or placebo 2 tablets bid [9 AM and 8 PM], metformin 500 mg 2 tablets bid [9 AM and 8 PM]).

Peripheral venous blood (9 AM, after 12 hours of fasting) and overnight (from 10 PM to 7 AM) urine samples was collected at baseline and at the end of the 24-week randomized treatment period, for the measurements of plasma DKK-1 and urinary 11-dehydro-TXB₂, respectively.

Biochemical Measurements

Fasting plasma glucose was measured according to the glucose oxidase method. The HbA_{1c} level was determined by automated high-performance liquid chromatography (HPLC).²¹ The homeostasis model assessment of insulin resistance (HOMA-IR) was performed as described by Matthews et al.²²

Total cholesterol, triglyceride, and high-density lipoprotein cholesterol concentrations were measured as previously described.²³ Low-density lipoprotein cholesterol was calculated using the Friedwald formula.

DKK-1 and sCD40L levels were measured by using ELISAs (all from R&D Systems). ADMA levels were measured in plasma samples by using a commercially available enzyme-immunometric assay (DLD Diagnostika). The intra-assay and interassay coefficients of variations were <10% for all ELISAs.

Urinary 11-dehydro-TXB₂ excretion rate was measured by a previously described radioimmunoassay method that has been validated by using different antisera and by comparison with gas chromatography–mass spectrometry.²⁴

Statistical Analysis

With 124 patients not receiving ASA, 90 receiving ASA, and 30 controls recruited, the study had 98% power to detect a 2-fold difference in plasma DKK-1 between patients with T2DM not treated with ASA versus controls and 95% power to detect a reduction by $\geq 50\%$ in ASA-treated diabetic patients versus diabetic patients not receiving ASA.

The Kolmogorov–Smirnov test was used to determine whether each variable had a normal distribution. When

necessary, log transformation was used to normalize the data, or appropriate nonparametric tests were used (Mann–Whitney *U*-test; Spearman correlation coefficient). Differences at baseline in categorical variables were analyzed by χ^2 or Fisher's exact test. Comparisons between groups were made with *t* test for independent samples or Mann–Whitney *U*-test. Comparisons within groups, preintervention versus postintervention, were made with the Wilcoxon test. A multiple linear regression analysis, with variables significantly related to DKK-1 at univariate analysis and a priori, clinically relevant potential confounders such as age, sex, fasting plasma glucose, HbA_{1c}, and DM duration as covariates, was performed to identify predictors of DKK-1. Each covariate was tested in its original form or transformed if needed. In addition, each variable included was tested for multicollinearity. Data are presented as mean (1 SD) or as median and 25th, 75th percentile. Only *P* values <0.05 were regarded as statistically significant. All tests were 2-tailed, and analyses were performed using a computer software package (or Statistical Package for the Social Sciences, version 18.0, SPSS Inc).

Results

Circulating DKK-1 in T2DM

Plasma DKK-1 levels were significantly higher in patients with T2DM than in healthy patients (median 2.0-fold increase, *P*<0.001; Figure 1A). Plasma ADMA and CD40L levels were also significantly higher in T2DM patients (*P*<0.0001; Figure 1B and 1C). Further, patients with T2DM not receiving ASA treatment displayed significantly higher DKK-1 levels than did patients with T2DM receiving ASA treatment (Figure 2A). As expected, urinary 11-dehydro-TXB₂ excretion rate was significantly lower in diabetic patients receiving ASA treatment compared with both diabetic patients not receiving ASA and healthy subjects (Figure 2B). In contrast, no significant difference was observed in plasma ADMA levels between patients receiving ASA and patients not receiving ASA (*P*=0.37; data not shown).

Determinants of Circulating DKK-1

Plasma DKK-1 levels were unrelated to any clinical parameter other than ongoing ASA treatment, including age, BMI, DM duration, fasting plasma glucose, HbA_{1c}, lipid profile, systolic or diastolic blood pressure, history of hypertension, dyslipidemia, or smoking, evidence of carotid stenosis on ultrasound, previous myocardial infarction, stroke or transient ischemic attack, and medications.

Because the large majority (79 of 90) of diabetic patients receiving chronic low-dose ASA therapy had a DM duration

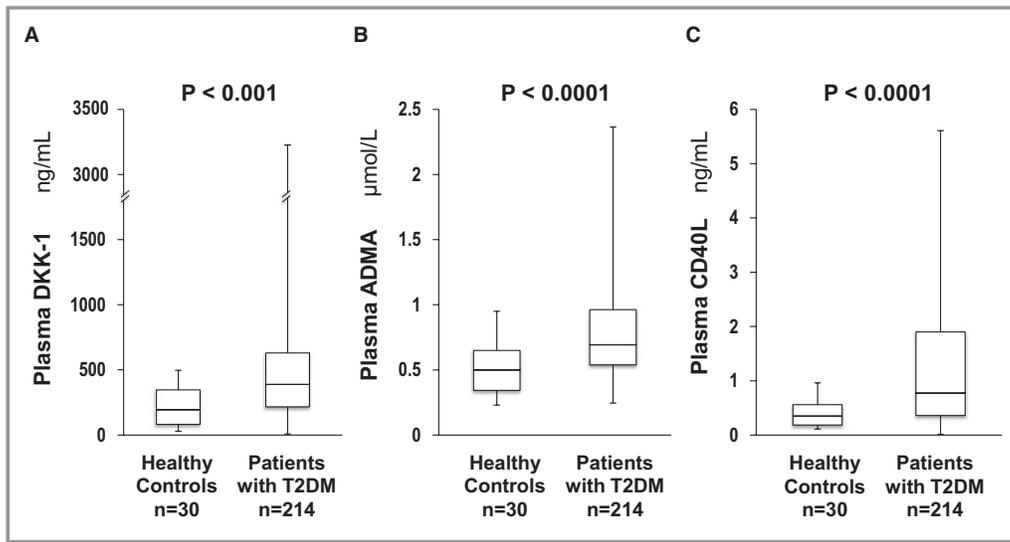


Figure 1. Plasma levels of DKK-1 (A), ADMA (B) and CD40L (C) in patients with type 2 diabetes mellitus (T2DM) and healthy controls. ADMA indicates asymmetric dimethylarginine; DKK-1, Dickkopf-1.

>1 year, we asked whether DM duration may affect DKK-1 levels and confound the interpretation of our findings about the impact of ASA treatment on plasma DKK-1. Thus, we analyzed the impact of DM duration within the subgroup of patients not receiving ASA (n=124), and no difference was detected in plasma DKK-1 concentration among patients with newly diagnosed (<1 year, n=54) versus long-standing (≥1 year, n=70) DM (P=0.902, Figure 3). In addition, among T2DM patients with long-standing DM, DKK-1 levels were not

different between patients with good (Hb_{A1c} <7%) and worse metabolic control (374.1 [213.8 to 672.7] versus 401.6 [178.4 to 613.5] ng/mL, P=0.863).

In patients not receiving ASA, DKK-1 was unrelated to any clinical or anthropometric variable. A significant correlation was found between plasma DKK-1 and urinary 11-dehydro-TXB₂, plasma ADMA and sCD40L levels in the whole cohort of patients not receiving ASA and controls (ρ=0.303, P<0.0001, ρ=0.45; P<0.0001, and ρ=0.37, P<0.0001, respectively)

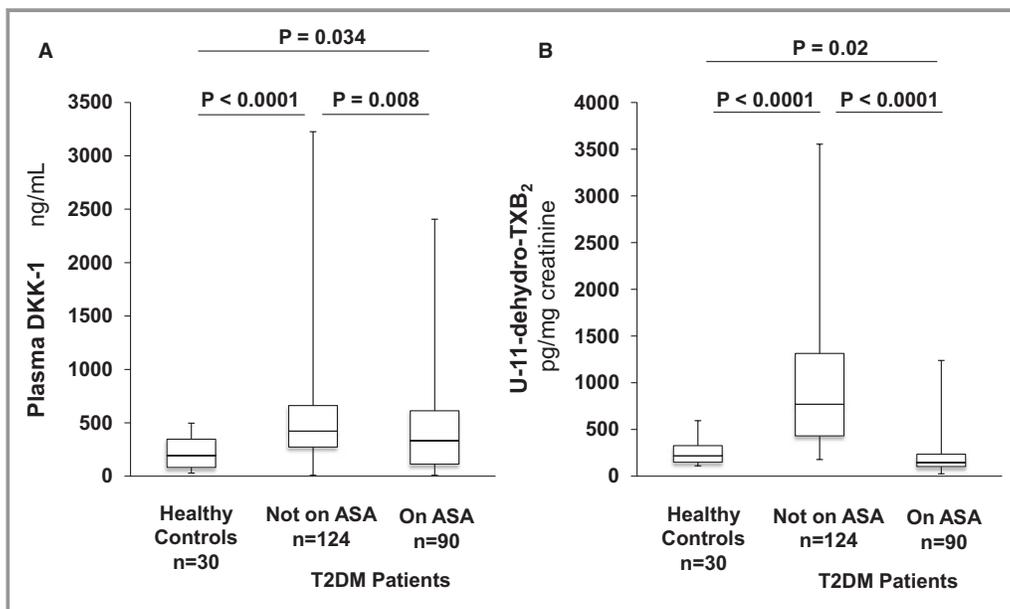


Figure 2. Plasma levels of DKK-1 (A) and urinary levels of 11-dehydro-TXB₂ (B) in healthy controls and in 2 groups of patients with type 2 diabetes mellitus (T2DM), according to ASA therapy. ASA indicates aspirin; DKK-1, Dickkopf-1.

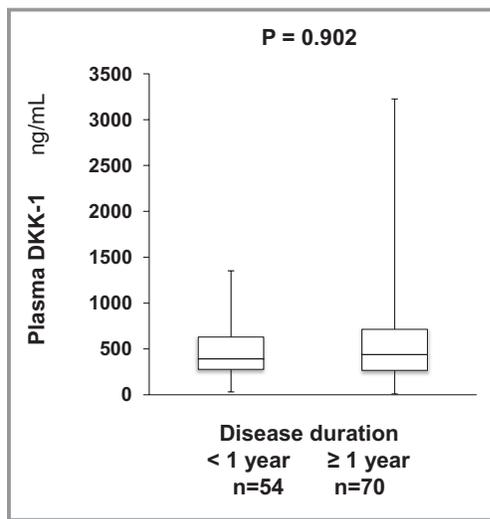


Figure 3. Plasma Dickkopf-1 concentration among patients not receiving ASA treatment with newly diagnosed (<1 year) vs long standing (≥ 1 year) diabetes.

(Figure 4 and data not shown). The significant correlation between plasma DKK-1 and urinary 11-dehydro-TXB₂, and plasma ADMA remained significant when evaluated in diabetic patients not receiving ASA treatment alone ($\rho=0.19$, $P=0.03$, and $\rho=0.33$, $P=0.006$, respectively, data not shown).

To further define the relationship between DKK-1, metabolic variables, atherosclerotic risk factors, and TX-dependent platelet activation in the 124 T2DM not treated with ASA and controls, a multiple regression analysis was performed at baseline in which DKK-1 was included as the dependent variable. Stepwise linear regression yielded a model in which only ADMA (regression coefficient=0.298, SEM=0.211, $P=0.013$) and urinary 11-dehydro-TXB₂ (regression coefficient=0.252, SEM=0.112, $P=0.033$) predicted DKK-1 levels, independent of age, sex, fasting plasma glucose, HbA_{1c}, and DM duration (adjusted $R^2=0.15$).

Effect of Metabolic Control on Circulating DKK-1 in T2DM

Acarbose

We next evaluated in a subgroup of patients with T2DM diagnosis within 1 year, not receiving ASA, whether improvement of metabolic control might be associated with concurrent changes in DKK-1 concentrations. A 20-week treatment with acarbose in 10 patients randomized to receive the active medication, was associated with a significant decrease in both fasting ($P=0.038$) and postprandial plasma glucose ($P=0.013$), and in HbA_{1c} ($P=0.025$), but not in HOMA-IR ($P=0.99$) and with a consistent reduction in DKK-1 ($P=0.005$)

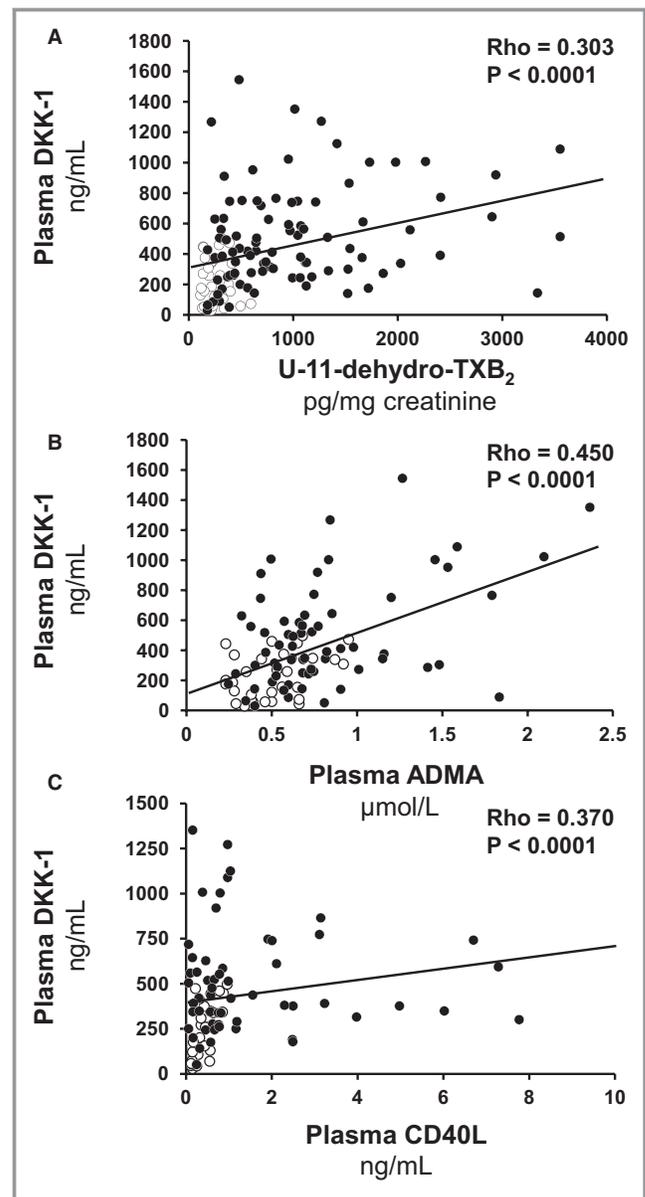


Figure 4. Correlations between plasma DKK-1, urinary 11-dehydro-TXB₂ (A), plasma asymmetric dimethylarginine (ADMA, B), and sCD40L (C) in patients with type 2 diabetes mellitus not receiving ASA therapy (n=124, solid circles) and healthy controls (n=30, open circles). DKK-1 indicates Dickkopf-1; sCD40L, soluble CD40L.

and urinary 11-dehydro-TXB₂ ($P=0.005$) levels. In contrast, placebo treatment was not associated with any significant change in any of the examined variables in the 10 patients randomized to placebo. In the whole group of subjects evaluated longitudinally, a significant direct correlation was observed between the change in plasma DKK-1 and the change in fasting plasma glucose ($\rho=0.53$, $P=0.013$), ADMA ($\rho=0.60$, $P=0.005$), and urinary 11-dehydro-TXB₂ ($\rho=0.67$, $P<0.0001$) (Figure 5), whereas no correlation of any biochemical variable with the change in the HOMA-IR was observed (data not shown).

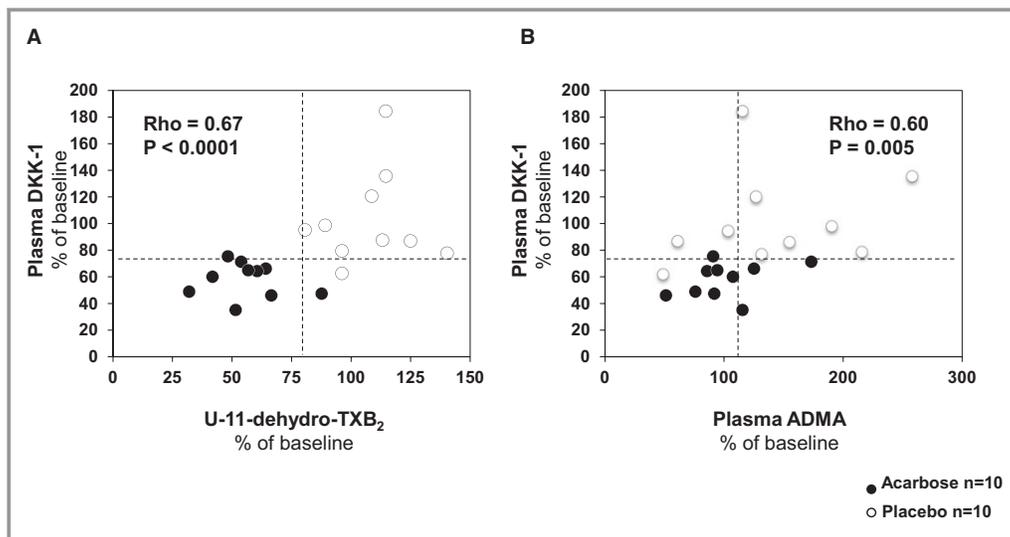


Figure 5. Correlations of individual percentage of baseline of plasma DKK-1, ADMA (A), and urinary 11-dehydro-thromboxane (TX)₂ (B), in a group of subjects with T2DM diagnosis within 1 year, not receiving ASA, evaluated longitudinally during 20 weeks of randomized acarbose (closed circles) vs placebo treatment (open circles). Vertical and horizontal dotted lines mark the boundaries of median values of percentage of baseline for both urinary metabolite excretion rates. ADMA indicates asymmetric dimethylarginine; DKK-1, Dickkopf-1; T2DM, type 2 diabetes mellitus.

Rosiglitazone

Finally, we evaluated the effect of improvement of metabolic control on DKK-1 plasma levels and TX-dependent platelet activation in patients with variable disease duration (1 to 10 years) and not receiving chronic ASA treatment. A 24-week treatment with rosiglitazone in 11 patients randomized to receive the active medication on top of metformin was associated with a significant decrease in both fasting plasma glucose ($P=0.003$) and in HbA_{1c} ($P=0.029$) and with a consistent reduction in DKK-1 ($P=0.004$) and urinary 11-dehydro-TXB₂ ($P=0.004$) levels. In the group of subjects randomized to rosiglitazone, evaluated longitudinally, a significant direct correlation was observed between urinary 11-dehydro-TXB₂ and both fasting plasma glucose ($\rho=0.439$, $P=0.041$) and plasma DKK-1 throughout the treatment period ($\rho=0.794$, $P<0.0001$) (Figure 6).

Discussion

Wnt signaling has previously been linked to regulation of glucose metabolism, through its involvement in normal pancreatic islet development, in pancreatic beta cell function and genesis, as well as in the production of the incretin hormone glucagon-like peptide 1 (GLP-1) and through its association with susceptibility genes for T2DM.¹¹ In the present study, we, to the best of our knowledge, for the first time show that patients with T2DM are characterized by markedly elevated circulating levels of the Wnt antagonist

DKK-1, which were associated with urinary levels of 11-dehydro-TXB₂, plasma ADMA, and plasma CD40L. Plasma DKK-1 levels were lower in T2DM patients treated with ASA and decreased during improvement of metabolic control with acarbose in a subgroup of patients. Together, these data suggest that DKK-1 levels in T2DM may reflect interactions between inflammation, endothelial dysfunction, and platelet activation.

In atherothrombotic diseases, the interaction between platelets and an activated endothelium may result in further enhanced platelet activation and thrombus formation.¹ In fact, platelets release the contents of their granules, and this platelet release includes a multitude of inflammatory substances, which can attract atherogenic leukocytes from the circulation and activate endothelial cells.¹ Thor Ueland et al described DKK-1 as a novel mediator in platelet-mediated endothelial cell activation.¹⁴ Their in vitro experiments identified a role for platelet- and endothelial-derived DKK-1 in platelet-dependent endothelial activation, promoting enhanced release of inflammatory cytokines. Thus, DKK-1 may enhance platelet-mediated endothelial cell activation involving the Wnt/ β -catenin and necrosis factor- κ B pathways. Moreover, neutralizing antibodies against DKK-1 abrogate platelet-induced cytokine production in endothelial cells and silencing endothelium-derived DKK-1 attenuates the inflammatory interaction between platelets and endothelial cells.¹⁴ Based on the demonstration of positive DKK-1 immunostaining in platelet aggregates at the site of plaque rupture in ST-segment elevation myocardial infarction patients as well as in the endothelium of

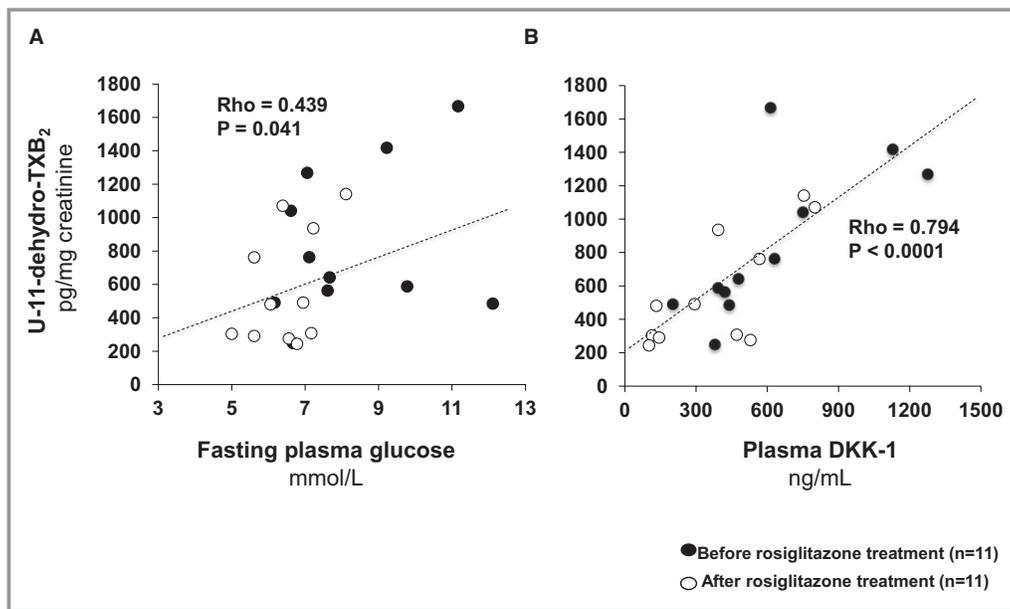


Figure 6. Correlations between urinary 11-dehydro-thromboxane B₂ and both fasting plasma glucose (A) and plasma Dickkopf-1 (B) throughout the 24-week rosiglitazone treatment period, in a subgroup of patients with variable disease duration (1 to 10 years) and not receiving chronic ASA treatment, randomized to rosiglitazone.

symptomatic carotid plaques,¹⁴ such inflammatory effects of DKK-1 could potentially also be operating in vivo within an atherosclerotic lesion. The demonstration of a major role of DKK1 in the angiogenic processes seems to confirm the link between the wingless family, the Wnt inhibitor, DKK1, and the endothelial function.²⁵

In our T2DM patients, the relationship between DKK-1, platelet activation markers, and ADMA levels seems to enforce the hypothesis of a possible key role of DKK-1 in mediating the inflammatory interaction between platelets and endothelial cells. The key role of DKK-1 in these processes, which are clearly relevant in relation to atherogenesis, is also supported by some clinical studies. Thus, DKK-1 is an independent predictor of long-term major adverse cardiac events in patients with ACS.²⁶ Previous studies have also shown a close association of DKK-1 levels and atherosclerotic diseases such as myocardial infarction or ischemic cerebrovascular disease.^{12,27} Moreover, circulating DKK-1 levels are inversely associated with coronary artery and aortoiliac calcified plaque and even with carotid intima-media thickness in T2DM and predict long-term mortality in patients with symptomatic aortic stenosis.^{28–31} In our cohort, less than 1 of 4 patients (23.8%) had a history or evidence of vascular disease, and circulating DKK-1 levels were unrelated to vascular events or carotid plaque, regardless of ongoing ASA treatment. The short sample size of patients with atherosclerotic vascular disease may have biased our analysis and deserves confirmation in larger samples. Moreover, future

prospective studies with long-term outcome will have to determine if the elevated levels of plasma DKK-1 found in T2DM patients in this study are associated with adverse events in this population.

In the past years, a growing body of evidence indicates a possible involvement of Wnt signaling in glucose metabolism and susceptibility to DM development.^{32,33} Recently, an experimental study demonstrated that inhibition of the Wnt antagonist DKK-2 reduces basal blood glucose concentrations and improves glucose tolerance, suggesting that targeting Wnt antagonism may be a treatment option in T2DM.³⁴ Indeed, our study demonstrates in vivo that DKK-1 is enhanced in the circulation of patients with T2DM, even in the earlier stages of the disease,² is associated with endothelial dysfunction and platelet activation, and is down-regulated by ameliorating glycemic control and/or by inhibiting a putative source of its release,³ namely platelets, by low-dose ASA treatment. These findings suggest that targeting DKK-1 in T2DM merits further investigation.

The lack of association between DKK-1 concentrations and measures of glucose metabolism in the whole group might be explained with the notion that Wnt signaling stimulates proglucagon and GLP-1 expression in enteroendocrine cells, and DKK2 deficiency does not cause an increase in insulin production but results in increased Wnt activity and GLP-1 production in the intestines.³⁴ Thereby, much of the effect of the DKK family on glucose tolerance might be largely indirect. While DKK family may adversely affect glucose tolerance, the

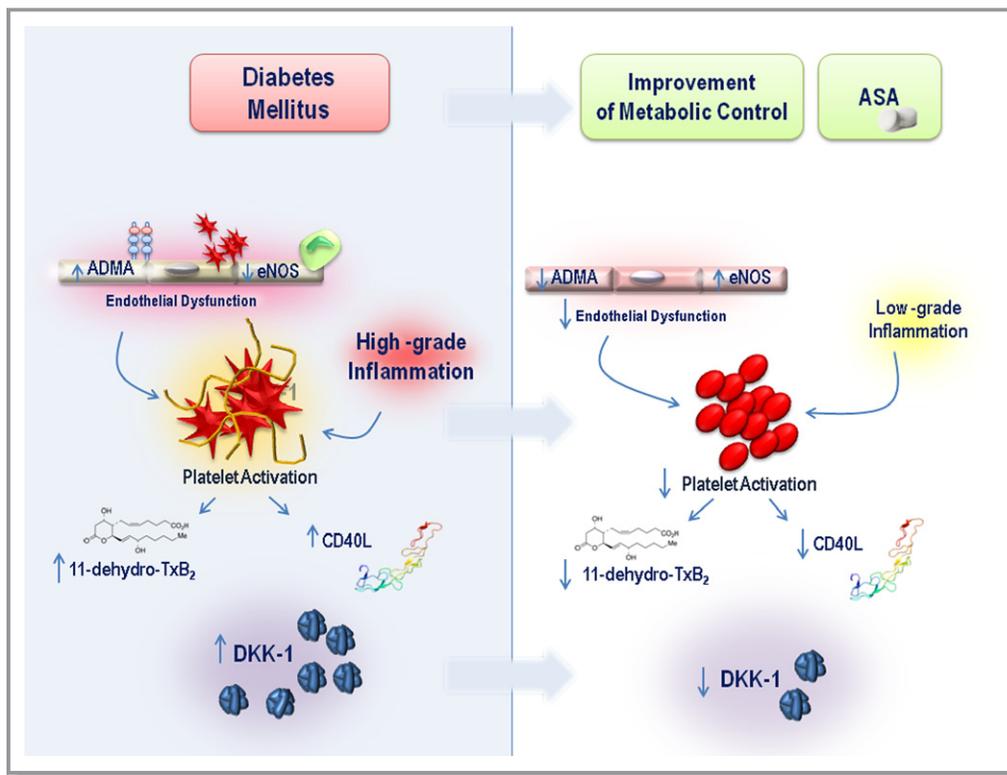


Figure 7. The illustration depicts the role of Wnt signaling and platelet-derived inflammatory signals (CD40L) in triggering endothelial dysfunction and persistent thromboxane-dependent platelet activation, as suggested by the experimental findings of the present study. It also illustrates potential amplification loops sustaining this mechanistic chain of events, as well as partial reversal by different interventions. ADMA indicates asymmetric dimethylarginine; ASA, aspirin; DKK-1, Dickkopf-1; eNOS, endothelial nitric oxide synthase.

latter may in turn modulate DKK concentrations, since amelioration of glycemic control exerted by acarbose down-regulates plasma DKK-1 levels. Such a significant reduction of DKK-1 with amelioration of glycemic control with acarbose and the linear correlation between the changes in the 2 parameters, coupled with lack of relationship with measures of insulin resistance, may suggest a prevalent intertwine of hyperglycemia over insulin sensitivity with circulating DKK-1. Of course, the small sample size does not allow us to draw definite conclusions from our findings.

The present study has some limitations such as the cross-sectional nature of the main study, not including an intervention with low-dose ASA: a time course of DKK-1 inhibition on ASA administration, consistent with the pattern of COX-1 inhibition, would have allowed definite conclusion about the cell origin of circulating DKK-1 and on the TX dependence of its release. In addition, the control group was not matched for age and sex with the cohort of patients with DM. Although DKK-1 plasma levels were not influenced by age and sex among T2DM, this limitation must be accounted for. Furthermore, lack of association between circulating DKK-1 levels and measures of glucose metabolism was based solely on *P* values and may

simply be due to low power. Finally, evaluation of the *in vivo* effects of GLP-1 analogs, not performed in this study, may gain further insight into the mechanisms linking DKK-1 and DM development and its complications. Finally, additional mechanistic studies are needed to further elucidate the role of Wnt signaling and DKK-1 in T2DM beyond the associations reported in the present study.

Together these data support the involvement of Wnt signaling even in the earlier phases of DM, with a potential contribution of DKK-1 in the inflammatory interaction between platelet activation and endothelial cells. To our knowledge, this study is the first to suggest that a circulating Wnt modulator may influence the atherothrombotic evolution of DM, involving at the same time glycemic control, inflammation, platelet activation, and endothelial dysfunction (Figure 7). However, prospective studies are needed to evaluate the pathogenic relevance of elevated Wnt antagonists in the setting of T2DM.

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Disclosures

None.

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Circulating Dickkopf-1 in Diabetes Mellitus: Association With Platelet Activation and Effects of Improved Metabolic Control and Low-Dose Aspirin

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