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BRIEF COMMUNICATION

Sodium-Glucose Co-Transporter 2 (SGLT2) Inhibitor Dapagliflozin Stabilizes Diabetes-Induced Atherosclerotic Plaque Instability

Yung-Chih Chen, PhD; Karin Jandeleit-Dahm, MD, PhD*; Karlheinz Peter, MD, PhD*

BACKGROUND: Diabetes is known to accelerate atherosclerosis and increase plaque instability. However, there has been a lack of suitable animal models to study the effect of diabetes on plaque instability. We hypothesized that the tandem stenosis mouse model, which reflects plaque instability/rupture as seen in patients, can be applied to study the effects of diabetes and respective therapeutics on plaque instability/rupture.

METHODS AND RESULTS: ApoE$^{-/-}$ mice at 7 weeks of age were rendered diabetic with streptozotocin and 5 weeks later were surgically subjected to tandem stenosis in the right carotid artery and fed with a high-fat diet for 7 weeks. As a promising new antidiabetic drug class, a sodium glucose co-transporter 2 inhibitor was tested in this new model. Diabetic mice showed an increase in the size of unstable atherosclerotic plaques and in the plaque instability markers MCP-1, CD68, and necrotic core size. Mice treated with dapagliflozin demonstrated attenuated glucose and triglyceride levels. Importantly, these mice demonstrated plaque stabilization with enhanced collagen accumulation, increased fibrosis, increased cap-to-lesion height ratios, and significant upregulation of the vasculoprotective NADPH oxidase 4 expression.

CONCLUSIONS: The tandem stenosis mouse model in combination with the application of streptozotocin represents a highly suitable and unique mouse model for studying plaque destabilization under diabetic conditions. Furthermore, for the first time, we provide evidence of plaque-stabilizing effects of sodium-glucose co-transporter 2 inhibitor. Our data also suggest that this newly developed mouse model is an attractive preclinical tool for testing antidiabetic drugs for the highly sought-after potential to stabilize atherosclerotic plaques.

Key Words: animal model ■ plaque instability ■ sodium glucose co-transporter 2 inhibitor ■ streptozotocin

Myocardial infarction is a major cause of mortality/morbidity in patients with diabetes and typically atherosclerosis is the underlying pathology. Diabetes has been postulated to accelerate the development of atherosclerosis and increase plaque instability/vulnerability, ultimately causing plaque rupture and myocardial infarction. Therapeutic approaches to stabilize vulnerable plaques in patients with diabetes are thus highly sought-after. Until recently there was no conclusive proof that any of the antidiabetic drugs provide cardiovascular protection. However, the advent of new antidiabetic agents, such as the sodium glucose co-transporter 2 inhibitor (SGLT2i) and glucagon-like peptide-1 receptor agonists, has substantially changed this scenario, adding certain antidiabetic drugs to the standard repertoire of cardiologists. This paradigm shift is based on several randomized, controlled clinical trials demonstrating that SGLT2i confer beneficial effects on cardiovascular outcomes in patients with type 2 diabetes.[1] There is clinical evidence that SGLT2i

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protects against heart failure; however, although postulated as a mechanism, there is no direct clinical proof of anti-atherosclerotic and/or plaque-stabilizing effects of SGLT2i.

We have previously validated a unique mouse model of plaque instability/rupture by introducing a tandem stenosis (TS) to the carotid artery.[2,3] This TS model closely reflects plaque instability/rupture as seen in patients[4] and has recently been successfully used to define the plaque-stabilizing effects of a myeloperoxidase inhibitor[5] and anti-CD47 antibodies.[6] We now systematically use this model to investigate whether diabetes promotes plaque instability and, most importantly, whether SGLT2i exhibit plaque-stabilizing effects.

METHODS

All experimental protocols and animal experiments have been approved by the Animal Ethics Committees of the Alfred Research Alliance approved number (E/1838/2018/B). The data sets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Animal Studies

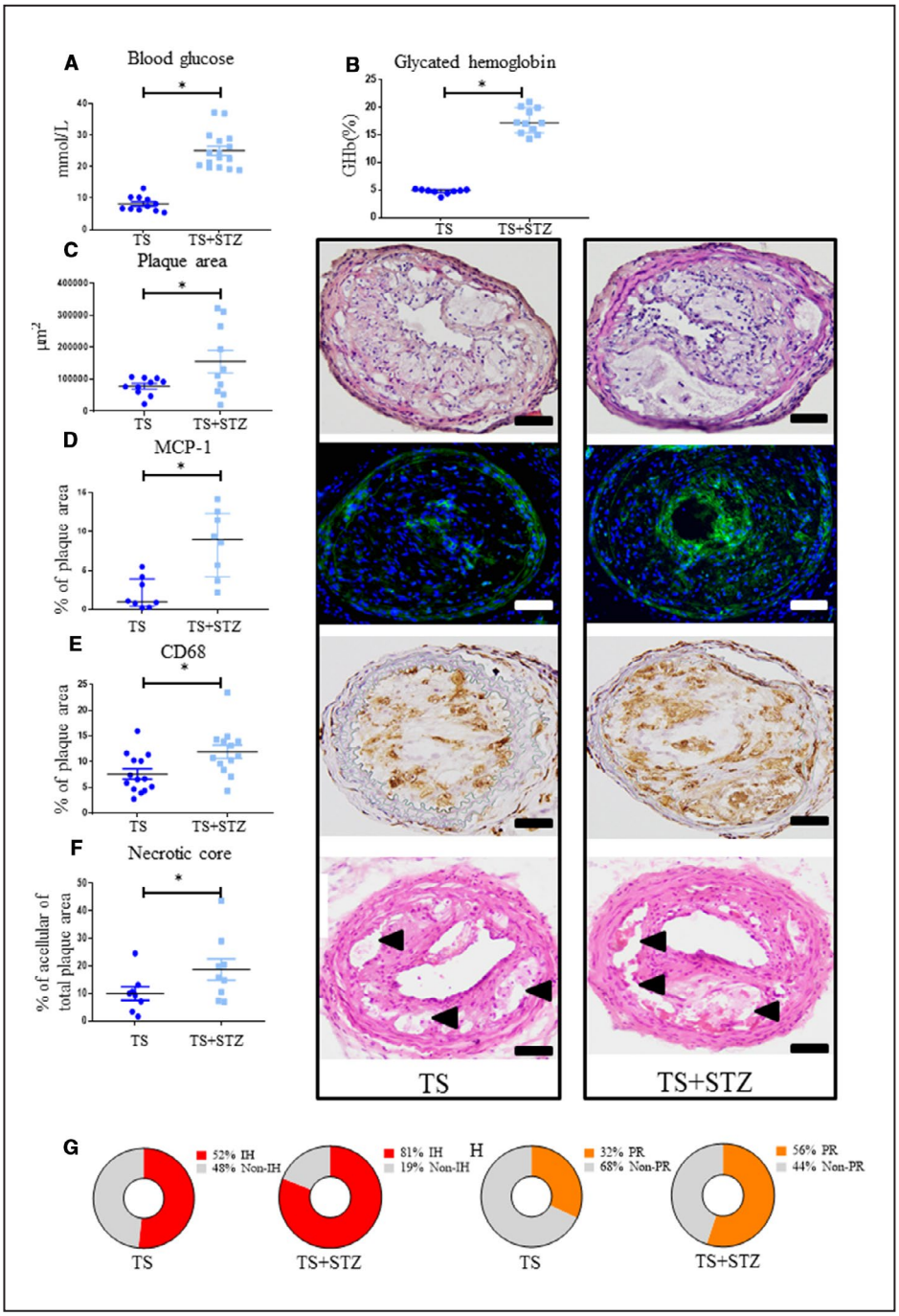
ApoE$^{-/-}$ mice of C57/Bl6 background (Australia Resource Centre, Western Australia) at 7 weeks of age were injected intraperitoneally with streptozotocin at a dose of 55 mg/kg for 5 consecutive days. At 12 weeks of age, 5 weeks after streptozotocin, mice were anesthetized intraperitoneally by a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). An incision was made in the neck and the right common carotid artery was dissected from circumferential connective tissues. TS with 150-µm outer diameters was introduced, with the distal point 1 mm from the carotid artery bifurcation and the proximal point 3 mm from the distal stenosis. The stenosis diameter was obtained by placing a 6–0 blue braided polyester fiber suture around the carotid artery together with a 150-µm needle that was tied to it and later removed as described previously.[2,5] The right carotid artery did not exhibit atherosclerotic plaques at the time point of the TS surgery. Animals were euthanized at 7 weeks after TS surgery, because this time point has been shown to be most sensitive to report on differences in plaque instability at different cardiovascular risk settings (eg, trimethylamine-rich diet) as well as testing of plaque-stabilizing drugs.[2,5,7]

Histology and Immunohistochemistry

The left and right common carotid arteries and brachiocephalic trunk were sectioned as described previously.[2] Serial cryosections were obtained at 100 µm proximal to the proximal suture in the right common carotid artery and from the brachiocephalic trunk and stored at −80 °C until staining was performed. Picrosirius red staining was performed to highlight and quantify collagen within the atherosclerotic plaque. Hematoxylin and eosin staining was used for necrotic core determination and atherosclerotic plaque burden. Oil red o staining was used for lipid content quantification in the plaque. Images were obtained using a light microscope (Olympus BX50). Sections were incubated with rat anti-mouse TER-119 biotin-labeled antibody (eBioscience 13-5921) 1:400; rat anti-mouse CD68 (AbD Serotech MCA1957GA) 1:100, or MOMA-2 (AbD Serotech MCA519G); rabbit polyclonal anti-NOX-4 (Sigma-Aldrich ABC459) 1:100; and anti-monocyte chemoattractant protein-1 antibody (Abcam ab7202) 1:200 at 4 °C overnight. After washing steps and incubation in ABC reagent, DAB reagent was applied to

Figure 1. Glucose, glycated hemoglobin, and atherosclerotic plaque area and composition in the newly developed diabetes-induced unstable plaque mouse model.

A, Streptozotocin-treated mice with TS showed upregulation of blood glucose levels compared with nondiabetic TS mice. **B**, Streptozotocin-treated mice showed an increase in glycated hemoglobin (GHb). Glycated hemoglobin A1c was measured by HPLC. **C**, Diabetic mice showed an increase of plaque area in the right carotid artery of the TS model. Quantification was performed on hematoxylin and eosin (H&E)-stained sections, where the plaque was measured from the lumen to internal elastic lamina. Each data point represents the mean of 6 sections per mouse at 200-µm intervals. Representative H&E stainings are shown on the right. **D**, MCP-1 was upregulated in diabetic mice. The percentage of positive MCP-1 staining per atherosclerotic plaque area was obtained. Each data point represents the mean of 2 sections per mouse. The green color indicates representative immunofluorescence staining of MCP-1 on the right. **E**, Unstable plaques in diabetic mice showed an increase in macrophage staining. Macrophages were detected by staining for CD68 and quantified in relation to plaque area. Representative examples are shown on the right. **F**, Unstable carotid plaques in diabetic mice showed an increase in necrotic core size. Quantification was performed in relation to plaque area. Typical examples are shown on the right with arrowheads indicating the necrotic, acellular area. **G**, Intraplaque hemorrhages (IH) were increased in diabetic mice. IH was defined by immunostaining of the red blood cell marker TER-119 or visible hemorrhage in the unstable carotid plaques area of the right carotid artery. IH in the TS and the TS+streptozotocin group were found in 60 out of 116 and 81 out of 100 mice, respectively. $P<0.0001$ by Fisher exact test. **H**, Plaque rupture (PR) was increased in diabetic mice. PR was defined by visible defects of the fibrous cap with or without luminal thrombi. PR in the TS and the TS+streptozotocin group was found in 25 out of 96 and 48 out of 87 mice, respectively. $P<0.0001$ by Fisher exact test. All other data represent N=13 to 16, mean±SEM, assessed with Student *t* test (**A**, **C**, **E**, and **F**) or median with interquartile ranges assessed with the Mann–Whitney *U* test (**B** and **D**). *$P<0.05$. Bars indicate 100 µm. HPLC indicates high-performance liquid chromatography; MCP-1, monocyte chemoattractant protein-1; STZ, streptozotocin; and TS, tandem stenosis.



each section. Conversion of the DAB substrate into a colored product was monitored and slides were immersed in dH_2O to stop the reaction. Sections were dehydrated and covered with coverslips using DPX mounting media. Quantification of images was performed on Fiji Image J.

Statistical Analysis

Quantitative variables are summarized as mean±SEM or median with interquartile ranges if non-normally distributed. Comparison between 2 groups was performed using the unpaired Student *t* test or Mann–Whitney test for non-normally distributed variables. Association between 2 categorical variables was determined using Fisher exact test. Comparisons among 3 groups were performed using 1-way ANOVA or the Kruskal–Wallis test for non-normally distributed variables, followed by post hoc analysis using Dunn's multiple comparisons test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

We induced insulin-deficient diabetes using the beta-cell toxin streptozotocin in ApoE$^{-/-}$ mice on a high-fat diet that underwent TS surgery after confirming diabetes. The mice with diabetes showed significantly elevated glucose and glycated hemoglobin levels (Figure 1A and 1B). Under diabetic conditions, the region previously in the TS model defined as exhibiting unstable atherosclerotic plaque (proximal to the first stenosis[2]) showed a significant increase in plaque area (Figure 1C). Importantly, several typical markers of plaque instability, such as the monocyte chemoattractant protein-1 (Figure 1D), macrophages (CD68; Figure 1E), and necrotic core size (Figure 1F), were significantly increased as quantified as the percentage of plaque area. As a direct proof of plaque instability, intraplaque hemorrhage increased from 52% in mice without diabetes to 81% in streptozotocin-treated diabetic mice (Figure 1G) and plaque rupture increased from 32% to 56%, respectively (Figure 1H, Figure S1) as measured in a total of 226 mice, whereby 116 were mice without diabetes with TS and 100 were streptozotocin-treated mice with diabetes with TS. These data confirm that diabetic conditions cause plaque instability and demonstrate the suitability of the TS mouse model for detecting diabetes-associated changes in plaque instability.

Next, we examined the potential of the SGLT2 inhibitor dapagliflozin to stabilize plaques. We induced diabetes with streptozotocin as described in Methods and dapagliflozin was given to ApoE$^{-/-}$ mice via drinking water 3 days after TS surgery. Two weeks after the start of dapagliflozin treatment, streptozotocin-induced hyperglycemia was modestly attenuated (Figure 2). The effect on lowering glucose was sustained for another 6 weeks; however, normoglycemia was never achieved. We focused on investigating markers of plaque instability. Interestingly, we did not see changes in inflammatory markers such as the macrophage markers (CD68 and MOMA-2) or monocyte chemoattractant protein-1 levels (Figure S2). Although the strongest markers of plaque instability such as plaque rupture and intraplaque hemorrhage were unchanged, early signs of plaque stabilization such as an overall reduction in lipid content (Figure 3A) and increases in collagen content and the cap-to-lesion height ratio were significantly changed in the dapagliflozin-treated group (Figure 3B and 3C). These data are of substantial translational relevance because the cap-to-lesion height ratio is a particularly important clinical marker that reflects plaque stability and is closely linked to improved clinical outcomes in patients.

Increased formation of reactive oxygen species is associated with plaque instability in diabetes. NADPH oxidases, particularly increased expression of NADPH oxidase 1 (NOX-1) and reduced expression of the vasculoprotective NOX-4 isoform in advanced plaques, have been shown to contribute to plaque development and remodeling in diabetes.[8] Interestingly, while expression of pro-atherosclerotic NOX-1 was not upregulated, the expression of anti-atherosclerotic NOX-4 (Figure 3D) was upregulated in the dapagliflozin group. Therefore, the regulation of certain NOX isoforms by SGLT2i therapy may represent a potential mechanistic explanation for the beneficial effect seen in patients treated with SGLT2i.

DISCUSSION

Accelerated coronary artery disease in patients with diabetes has become the leading cause of premature mortality and increased morbidity worldwide. There is an unmet need to study the mechanisms of diabetes-accelerated atherosclerosis and to identify novel therapeutic targets and strategies. Although several diabetic atherosclerosis mouse models have been studied, these animal models have limitations in mimicking all aspects of human diabetes-associated atherosclerosis. It is paramount that a translationally relevant mouse model of diabetes-associated atherosclerosis progresses to advanced, unstable atherosclerotic plaques undergoing plaque rupture. An ideal model will also have to respond to pharmacological interventions as seen in humans. Here we demonstrate the first plaque instability/rupture mouse model in the context of diabetes-accelerated atherosclerosis in association with key makers of plaque instability such as increased intraplaque hemorrhage and a decreased cap-to-lesion height ratio.

SGLT2 inhibitors have emerged as a new therapeutic class for lowering blood glucose. Several clinical trials have demonstrated the efficacy of the glucose-lowering effect of SGLT2i (EMPA-REG OUTCOME [Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients]; CANVAS [Canagliflozin Cardiovascular Assessment Study]) in association with improved cardiovascular outcomes. The EMPA-REG and CANVAS studies reported positive results with respect to the primary end point (major adverse cardiovascular events), but neutral effects were shown in the DECLARE-TIMI53 (Multicenter Trial to Evaluate the Effect of Dapagliflozin on the Incidence of Cardiovascular Events).[9,10] The EMPA-REG OUTCOME trial of empagliflozin demonstrated a reduction in cardiovascular outcomes and death from other causes.[10] Empagliflozin also reduced first and recurrent clinical events in patients with type 2 diabetes with atherosclerotic cardiovascular disease in a secondary analysis of the EMPA-REG outcome trial.[11] A post hoc analysis of the CANVAS Program and CREDENCE (Evaluation of

the Effects of Canagliflozin on Renal and Cardiovascular Outcomes in Participants With Diabetic Nephropathy) trial interestingly suggested a reduction of non-ST-segment–elevation myocardial infarctions and an increase in ST-segment–elevation myocardial infarctions, which suggests a differential effect on plaque instability in patients with non–ST-segment–elevation myocardial infarction and ST-segment–elevation myocardial infarction by SGLT2i.[12] Nevertheless, direct evidence that SGLT2i stabilizes atherosclerotic plaques is still lacking, because animal models of atherosclerosis typically do not exhibit plaque instability and rupture. Furthermore, it is not known whether all SGLT-2 inhibitors are similarly effective in promoting plaque stabilization. Currently, only a few experimental studies have investigated the effect of SGLT2i in classical mouse models of atherosclerosis. Al-Sharea et al treated LDLR$^{-/-}$ mice (on a high-fat diet and after streptozotocin application) with dapagliflozin and reported a reduction in peripheral monocyte count and a lower degree of atherosclerosis, postulating a change in plasma lipoproteins as the major cause.[13] In ApoE$^{-/-}$ mice on a high-fat diet, empagliflozin reduced blood glucose, aortic plaque area, insulin resistance, and inflammation.[14]

The current study demonstrates the plaque-stabilizing capability of dapagliflozin associated with an increased cap-to-lesion height ratio, reduced intraplaque lipid accumulation, and increased plaque collagen content. These changes were associated with a significant reduction of serum glucose and triglyceride levels (Figure S3). Dapagliflozin treatment did not significantly attenuate macrophage infiltration or monocyte chemoattractant protein-1 expression and had no effect on overall plaque area (Figure S2). Discrepant effects on plaque area and composition in the various parts of the aorta have previously been described with inhibitors of advanced glycation end products.[15] Interestingly, dapagliflozin seems to be more effective on fibrosis and remodeling of plaques rather than on inflammatory parameters. However, we demonstrated reduced NOX-4 expression in diabetic TS mice and increased plaque NOX-4 expression with dapagliflozin treatment. This is consistent with findings in advanced plaques in mice and in atherosclerotic plaques of patients with diabetes and cardiovascular diseases.[8] We have previously demonstrated a vasculoprotective effect of vascular NOX-4.[8] Complete genetic NOX-4 deletion in diabetic ApoE$^{-/-}$ mice was associated with increased plaque area, increased macrophage infiltration and inflammation, as well as increased expression of newly formed collagens (collagen I and III) and reduced expression of collagen IV in the fibrous cap, suggesting that NOX-4 is at least in part involved in the plaque-stabilizing vascular remodeling process conferred by dapagliflozin treatment.

CONCLUSIONS

In conclusion, the TS mouse model in conjunction with streptozotocin-induced diabetes reflects plaque destabilization as seen in patients with diabetes. Importantly, our studies provide initial proof-of concept for the

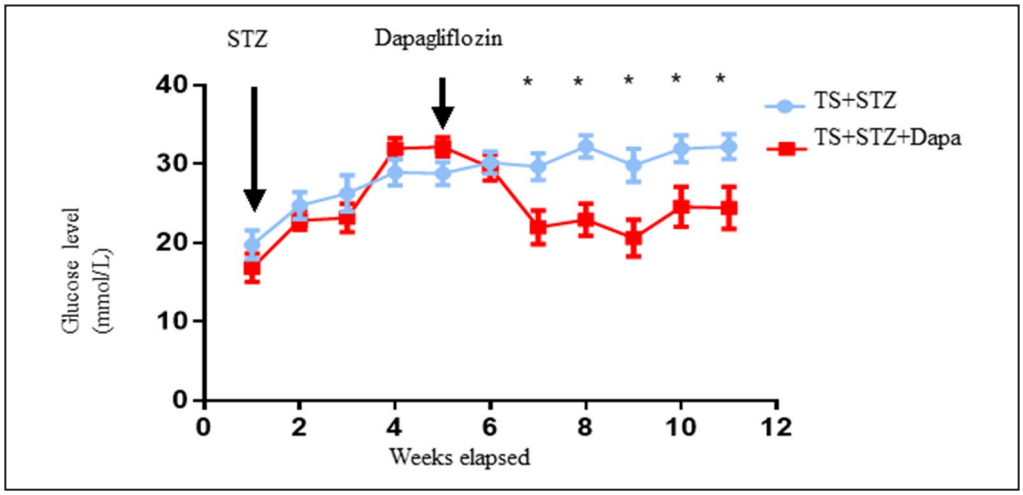


Figure 2. Comparison of weekly blood glucose measurements with and without SGLT2 inhibitor dapagliflozin treatment.
The SGLT2 inhibitor, dapagliflozin, reduced hyperglycemia in STZ-treated diabetic TS mice. Dapagliflozin was given in drinking water at 25 mg/kg after TS. Two weeks after the initiation of daily dapagliflozin, glucose levels were significantly reduced to 24.66±1.43 mmol/L compared with vehicle-treated diabetic TS mice with 28.61±1.13 mmol/L and remained steady until the end point. N=20 mice in the TS+STZ+Dapa cohort, N=23 mice in the TS+STZ cohort, mean±SEM, *P<0.05 by multiple t test. SGLT2 indicates sodium-glucose co-transporter-2; STZ, streptozotocin; and TS, tandem stenosis.

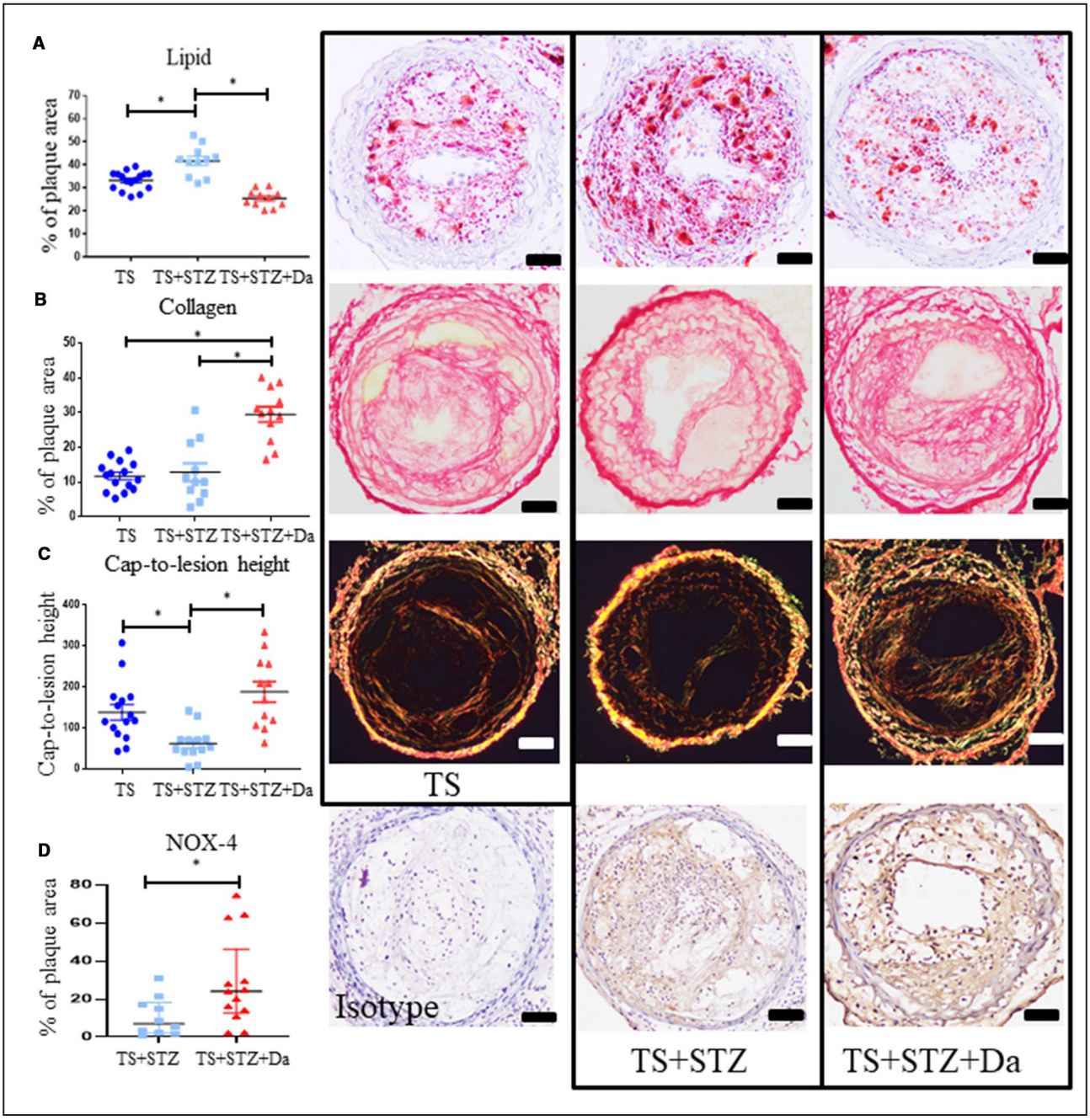


Figure 3. Atherosclerotic plaque composition is altered after SGLT2i treatment in the newly developed diabetes-induced unstable plaque mouse model.

Dapagliflozin (**A**) significantly reduced the lipid content and (**B**) increased the collagen content in the unstable atherosclerotic plaque area in the TS model. Oil red O staining and Picro-Sirius Red staining were used for quantifying the total lipid and collagen content, respectively. Representative examples are shown on the right. **C**, Diabetes significantly reduced the cap-to-lesion height ratio and dapagliflozin rescued this effect by increasing the cap-to-lesion height ratio, indicating a more stable plaque phenotype. The cap-to-lesion height ratio was determined by dividing the total fibrous cap area with the maximal lesion height in every cross-section. N=13 to 16, mean±SEM, **P*<0.05 by 1-way ANOVA with Dunn's multiple comparisons test. Representative examples are shown on the right. **D**, Dapagliflozin increased NOX-4 expression in unstable plaques of the right carotid artery. NOX-4 expression in plaques was determined by immunohistochemistry using an anti-NsOX-4 antibody and each data point represents the mean of 2 sections per mouse. Representative examples including an isotype control are shown on the right. N=13 to 16, data are presented as median with interquartile ranges as assessed by the Mann–Whitney *U* test. **P*<0.05. Bars indicate 100 μm. Da indicates dapagliflozin; NOX-4, NADPH oxidase 4; SGLT2i, sodium-glucose co-transporter-2 inhibitor; STZ, streptozotocin; and TS, tandem stenosis.

plaque-stabilizing effects of SGLT2i. Indeed, these effects on plaque stability could explain at least in part the reduction of cardiovascular events seen in patients with diabetes treated with SGLT2i and as such our findings bear direct translational relevance. Our data also establish the TS mouse model in combination with streptozotocin treatment as a preclinical tool for testing of antidiabetic drugs for highly sought-after plaque-stabilizing effects.

ARTICLE INFORMATION

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Author contributions: Chen collected and analyzed and interpreted the data. Chen performed the histological examination of the atherosclerotic plaques. Chen, Peter, and Jandeleit-Dahm co-designed the animal experiment and discussed the data, and jointly wrote the manuscript. All authors have read and have approved the final manuscript.

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Disclosures

None.

Supplemental Material

Figures S1–S3

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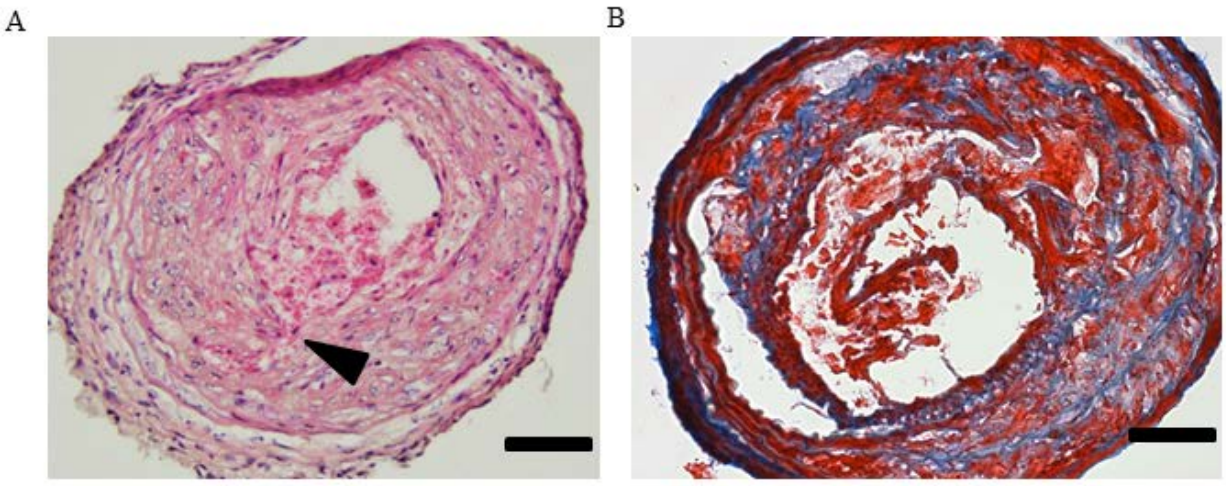
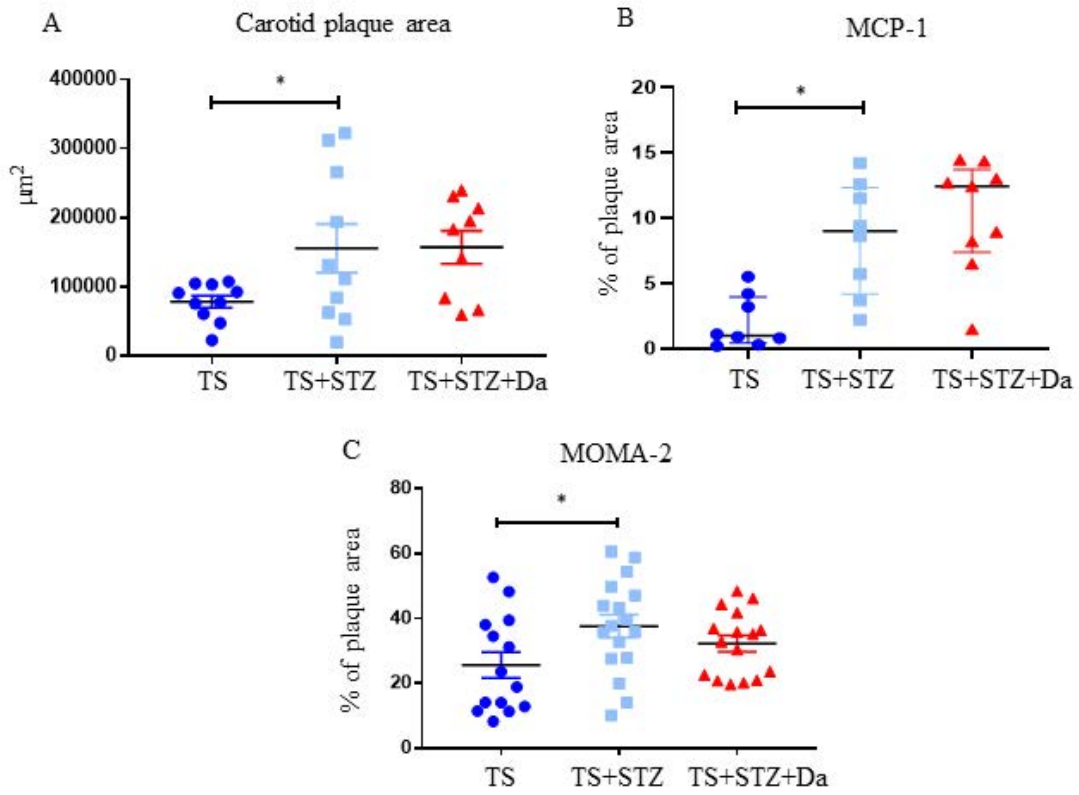
SUPPLEMENTAL MATERIAL

Figure S1. Representative histology of plaque rupture in the newly developed diabetes-induced unstable plaque mouse model.



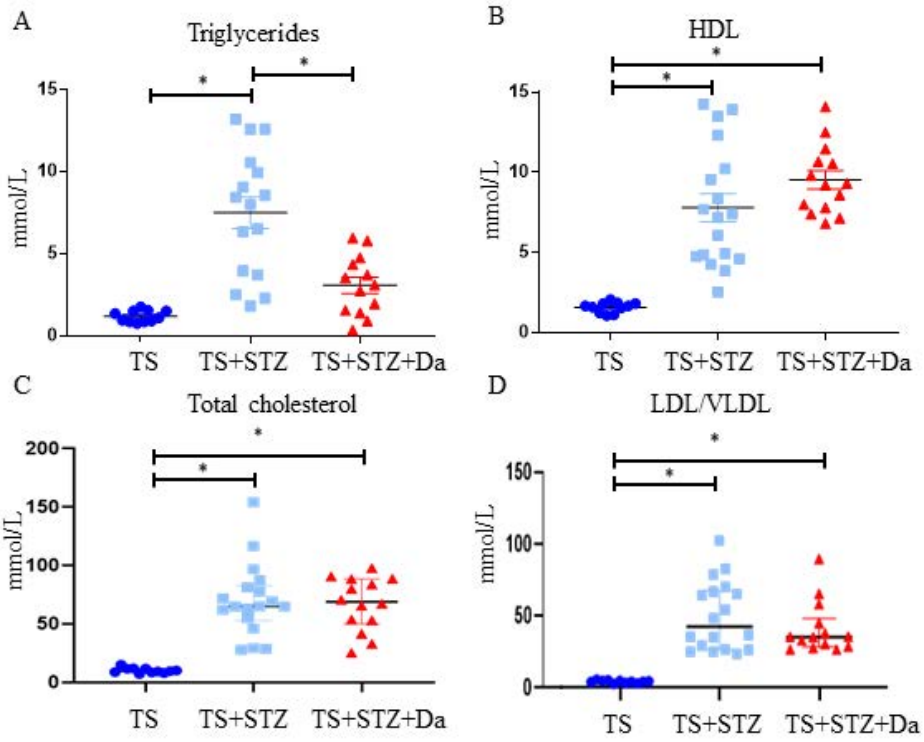
(A) Example of a plaque (cap) rupture in the unstable plaque area of a TS mouse treated with STZ with a luminal thrombus. A representative H&E staining is shown. (B) Example of a plaque rupture in the unstable plaque area of a TS mouse treated with STZ without a luminal thrombus. A representative Martius Scarlet Blue staining is shown, with fibrin represented in red. Bars indicate 100μm. STZ: Streptozotocin. TS: Tandem Stenosis.

Figure S2. Atherosclerotic plaque characteristics of SGLT2i treatment in the newly developed diabetes-induced unstable plaque mouse model.



STZ-treated diabetic TS mice show an increase of (A) plaque area, (B) MCP-1 and (C) macrophage staining. Treatment with dapagliflozin did not affect these changes. Each data point represents the mean value of six sections per mouse for H&E and two sections per mouse for immunofluorescence of MCP-1 and MOMA-2. N=13-16, data are presented as mean±S.E.M (A, C) or median with interquartile ranges (B), *$p<0.05$ as assessed by one-way ANOVA (A, C) or Kruskal-Wallis (B) with Dunn's multiple comparisons test. STZ: Streptozotocin. TS: Tandem Stenosis. Da: Dapagliflozin. SGLT2: Sodium-Glucose Cotransporter-2.

Figure S3. Systemic lipid profile in the newly developed diabetes-induced unstable plaque mouse model.



(A) Plasma triglycerides, (B) high density lipoproteins (HDL), (C) total cholesterol, and (D) low density lipoproteins/very low density lipoproteins (LDL/VLDL) were increased in STZ-treated diabetic TS mice, compared to mice with TS surgery only. Treatment with dapagliflozin reduced the elevated plasma triglyceride levels, while HDL, total cholesterol and LDL/VLDL remained unchanged. N=18-28, data are presented as mean±S.E.M. (A, B) and median with interquartile ranges (C, D), *$p<0.05$ as assessed by one-way ANOVA (A, B) or Kruskal-Wallis (C, D) with Dunn's multiple comparisons test. STZ: Streptozotocin. TS: Tandem Stenosis. Da: Dapagliflozin. SGLT2: Sodium-Glucose Cotransporter-2.