SGLT-2 inhibition by empagliﬂozin has no effect on experimental arterial thrombosis in a murine model of low-grade inﬂammation

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Aims

Low-grade inﬂammation couples dysmetabolic states to insulin resistance and atherosclerotic cardiovascular (CV) disease (ASCVD). Selective sodium–glucose co-transporter 2 (SGLT-2) inhibition by empagliﬂozin improves clinical outcomes in patients with ASCVD independently of its glucose lowering effects. Yet, its mechanism of action remains largely undetermined. Here, we aimed to test whether empagliﬂozin affects arterial thrombus formation in baseline (BSL) conditions or low-grade inﬂammatory states, a systemic milieu shared among patients with ASCVD.

Methods and results

Sixteen-week-old C57BL/6 mice were randomly assigned to acute administration of empagliﬂozin (25 mg/kg body weight) or vehicle, of which a subgroup was pre-treated biweekly over 4 weeks with super-low-dose lipopolysaccharide (LPS; 5 ng/kg body weight), before carotid thrombosis was induced by photochemical injury. The between-group difference in Doppler-probe detected time-to-occlusion remained within the predeﬁned equivalence margin (Δ = | 0.50|), irrespective of low-grade inﬂammation (95% conﬁdence interval, −9.82 to 8.85 and −9.20 to 9.69), while glucose dropped by 1.64 and 4.84 mmol/L, respectively. Ex vivo platelet aggregometry suggested similar activation status, corroborated by unchanged circulating platelet-factor 4 plasma levels. In concert, carotid PAI-1 expression and tissue factor (TF) activity remained unaltered upon SGLT-2 inhibition, and no difference in plasma D-dimer levels was detected, suggesting comparable coagulation cascade activation and ﬁbrinolytic activity. In human aortic endothelial cells pre-treated with LPS, empagliﬂozin neither changed TF activity nor PAI-1 expression. Accordingly, among patients with established ASCVD or at high CV risk randomized to a daily dose of 10 mg empagliﬂozin signatures of thrombotic (i.e. TF) and ﬁbrinolytic activity (i.e. PAI-1) remained unchanged, while plasma glucose declined signiﬁcantly during 3 months of follow-up.

Conclusion

SGLT-2 inhibition by empagliﬂozin does not impact experimental arterial thrombus formation, neither under BSL conditions nor during sustained low-grade inﬂammation, and has no impact on proxies of thrombotic/ﬁbrinolytic activity in patients with ASCVD. The beneﬁcial pleiotropic effects of empagliﬂozin are likely independent of pathways mediating arterial thrombosis.
Graphical Abstract

Acute administration of the sodium–glucose co-transporter 2 inhibitor empagliflozin and vehicle equivalently affect arterial thrombosis in a murine experimental model.

Keywords Arterial thrombosis • Empagliflozin • SGLT-2 inhibitors • Cardiovascular disease • PAI-1 • Tissue factor

1. Introduction

Mechanisms underlying atherosclerotic CV disease (ASCVD) intertwine with several factors including ageing, diabetes and obesity, and dominate morbidity and mortality worldwide. Chronic low-grade inflammation has emerged as a key mediator that couples obesity-induced dysmetabolism to insulin resistance, and mechanistically contributes to the high prevalence of ASCVD among these patients.1–6 Over decades, our understanding of CV complications linked to diabetic states was widely dominated by a ‘glucocentric’ view,7 which is now increasingly challenged as evidence from contemporary studies accumulates.8–11 Indeed, while microvascular lesions can be largely prevented by glucose lowering,12 the high propensity of diabetics for the build-up of atherosclerotic plaques with its commonly associated adverse events, such as myocardial infarction and stroke, operates independently of glucose levels, mainly involving inflammatory mechanisms.13–16

Sodium–glucose co-transporter-2 (SGLT-2) belongs to the sodium-dependent glucose co-transporter family and is predominantly expressed in the proximal tubule of the nephron, where it controls glucose reabsorption back into the circulation, thereby contributing up to 90% of the kidney’s glucose reabsorption capacity.17 Therefore, pharmaceutical blockage of SGLT-2 function by gliflozins results in blood glucose lowering, and represents a highly effective anti-diabetic remedy. A huge body of experimental evidence suggests that, beyond glycaemic control, SGLT-2 inhibitors may exert ‘off-target’ effects by modulating the activation of the arterial endothelium and improving endothelial dysfunction, thus blunting the evolution of atherosclerotic plaques.18 In fact, a recently published report highlights anti-inflammatorv and anti-oxidant effects on the endothelium conferred by empagliflozin, even when administered acutely.19 Yet, the effects of empagliflozin on acute atherothrombotic events during sustained low-grade inflammation, as it typically occurs during dysmetabolic states,5,14,15 is currently unknown.

Of all Food and Drug Administration-approved gliflozins, empagliflozin shows the highest selectivity for SGLT-2,20 and has been shown to improve CV outcomes in diabetic patients with established ASCVD,
Empagliflozin and arterial thrombosis

likely involving multiple mechanisms beyond glycaemic control. Indeed, among patients recruited in the EMPA-REG OUTCOME Trial\(^8\) those in the empagliflozin group experienced a relative risk reduction of the primary outcome (i.e. composite measure of death from CV causes, non-fatal myocardial infarction, or non-fatal stroke) by 14%, an effect that was consistent across the broad range of CV risk.\(^{21}\) Yet, no between-group difference in the frequency of non-fatal stroke and non-fatal myocardial infarction could be established during follow-up (FUP) (3.2 vs. 2.6%, \(P = 0.16; 4.5 \text{ vs. } 5.2\%\), \(P = 0.22\)). In concert, dapagliflozin-mediated SGLT-2 inhibition in diabetics recruited in the DECLARE–TIMI 58 Trial\(^9\) reduced the composite measure of CV death or hospitalization for heart failure by 17%, but failed to reduce the risk for myocardial infarction or ischaemic stroke. Similarly, in the integrated CANVAS Program,\(^11\) in which canagliflozin was used, SGLT-2 inhibition did not reduce the risk for non-fatal myocardial infarction or non-fatal stroke, collectively indicating that benefits of SGLT-2 inhibition are largely driven by reductions in mortality and/or hospitalization for heart failure, rather than acute atherothrombotic events.

Hence, we sought to investigate whether acute SGLT-2 inhibition by empagliflozin has indeed neutral effects on arterial thrombus formation in an established mouse model of endothelial-specific photochemical injury. In a separate set of experiments, before thrombosis induction, chronic low-grade inflammatory state was induced by super-low-dose lipopolysaccharide (LPS) administration, in an attempt to mimic the inflammatory milieu present in patients at high CV risk,\(^15\) such as those enrolled in the EMPA-REG OUTCOME Trial\(^8\) and the majority of patients recruited in the recently published EMPEROR-Reduced Trial.\(^10\)

2. Methods

2.1 Animals and treatment

In a first set of experiments 16-week-old C57BL/6j male mice were randomly subjected to receiving empagliflozin (Cayman Chemical Company, Ann Arbor, MI, USA) at the dose of 25 mg/kg bodyweight in 1:1 solution of 0.9% saline and polyethylene glycol (PEG) i.p., or vehicle (1:1 solution of 0.9% saline and PEG), 24 and 1 h before thrombosis was induced in the common carotid artery (CCA) (Figure 1A). Dosage and timing for empagliflozin administration were derived from previously published protocols.\(^{19,23}\)

A second batch of experiments was set to induce subclinical low-grade inflammation similar to patients at high risk for adverse CV events, as previously described.\(^1\) Here, 12-week-old C57BL/6j male mice were treated biweekly over 4 weeks with super-low-dose LPS (Sigma–Aldrich, St Louis, MO, USA; 5 ng/kg body weight in 0.9% saline i.p.). Then, 16-week-old mice were randomly subjected to receiving empagliflozin or vehicle, as described above, before undergoing in vivo carotid artery thrombosis. In this set of experiments, the first administration of empagliflozin concurred with the last administration of LPS (Figure 2A).

All animals were kept in a temperature-controlled animal facility under normal light/dark cycle with free access to food and water during the whole duration of the experiments. All procedures were approved by the Cantonal Veterinary Authority, Switzerland. Animal experiments were performed conforming to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

2.2 In vivo carotid artery thrombosis model

Arterial thrombosis was induced by photochemical injury of the CCA as previously described.\(^{24,25}\) Briefly, mice were anaesthetized by i.p. injection of 87 mg/kg sodium pentobarbital (Butler, Columbus, OH, USA). The depth of anaesthesia was confirmed by the absence of twitch reflex. Rose Bengal (Fischer Scientific, Fair Lawn, NJ, USA) was diluted to 12 mg/mL in phosphate-buffered saline and then injected through the tail vein at a concentration of 63 mg/kg. Mice were placed in supine position under a dissecting microscope and the right CCA was exposed by a midline cervical incision. A Doppler-flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY, USA) was applied and connected to a flowmeter (Model T106, Transonic Systems). Five to ten minutes after Rose Bengal injection, a 1.5 mW green light laser (540 nm; Melles Griot, Carlsbad, CA, USA) was directed to the site of injury at a distance of 6 cm from the artery for 60 min or until thrombosis occurred. From the onset of injury, carotid blood flow and heart rate were continuously monitored up to 120 min. Occlusion was defined as blood flow below 0.1 mL/min for at least 1 min. Cyclic flow variations were recorded and thrombus embolization was defined as an increase of blood flow to above 0.1 mL/min after previous decrease below said level lasting less than 1 min. At the end of the experiment, animals were euthanized by exsanguination through cardiac puncture under anaesthesia.

2.3 Ex vivo platelet aggregometry

Total blood cell count was performed on a ScilVet ABCplus (Horiba, Kyoto, Japan) using 3.8% citrate-anticoagulated blood. For platelet aggregation studies, washed platelets were obtained from citrate-anticoagulated blood drawn terminally by cardiac puncture in animals deeply anesthetized with isoflurane (5%), as previously described.\(^{26}\) Thereafter, washed platelets were re-suspended in Tyrode’s solution \([134 \text{ mM NaCl}; 0.34 \text{ mM Na}_{2} \text{HPO}_4; 2.9 \text{ mM KCl}; 12 \text{ mM NaHCO}_3; 20 \text{ mM Heps}; 5 \text{ mM glucose}; 0.35\% (w/v) bovine serum albumin; pH 7.0]\) and platelet counts were normalized to 200 000/μL. Platelets were activated with collagen (CRP-XL, Cambcol Lab., Cambridgeshire, UK; 10 μg/mL) or thrombin (Sigma–Aldrich, Buchs, Switzerland; 0.2 U/mL). Maximal aggregation (%), lag phase (s), and slope of aggregation (%/min) were assessed using light transmission aggregometry (PAAPT 4004 aggregometer, Haemochrom Diagnostica GmbH, Essen, Germany).

2.4 Determination of tissue factor activity and expression in arterial samples

Carotid arteries were lysed (50 mmol/L Tris–HCl, 100 mmol/L NaCl, 0.1% Triton X-100, pH 7.4), and total protein concentration was determined by Bradford protein assay according to the manufacturer’s recommendations (WVR Life Science AMRESCO, Solon, OH, USA). Tissue factor (TF) activity was determined as previously described by colorimetric ACTICHROME® TF assay (Cat No 846, American Diagnostica, Stamford, CT, USA).\(^{25,27}\) Briefly, arterial lysates were mixed with factor Vila and X following the manufacturer’s instructions, which lead to the conversion of factor X to Xa; factor Xa subsequently cleaves the chromogenic substrate SPECTROZYME FXa. Optical density of cleaved SPECTROZYME FXa was determined at 490 nm by Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and subtracted from absorbance at 405 nm. Finally, TF (pM) content was derived, according to a standard curve. TF activity as detected by the colorimetric assay was normalized to the total protein content of the sample and expressed as pmol/g of total protein. TF arterial expression was instead measured in arterial lysates by colorimetric enzyme-linked immunosorbent assay (ELISA; DY3178-05, R&D system, Minneapolis, MO, USA) and normalized to the protein concentration.
2.5 Plasma sampling for glucose, d-dimer, PAI-1, and platelet-factor 4 levels

Blood was collected via intracardiac puncture and immediately mixed with EDTA. The EDTA–blood solution was then centrifuged for 15 min at 3000 g as previously described.28 Plasma was collected and immediately snap-frozen in liquid nitrogen. Glucose levels were assessed by glucometer (Roche, Rotkreuz, Switzerland). Protein levels were measured by ELISA following the manufacturers’ instructions. D-dimer: abx258705, Abbexa, Cambridge, UK; PAI-1, DY3828-05, R&D Systems; platelet-factor 4 (PF4), MCX400, R&D Systems.

2.6 Cell culture experiments

Primary human aortic endothelial cells (HAECs) (Lonza, Basel, Switzerland) were cultured as previously described.29,30 Briefly, adhering HAECs were grown to confluence in fibronectin-coated 75 cm² flasks in endothelial growth medium (EGM-2, Lonza) supplemented with 10% foetal bovine serum (FBS). Cells were detached by using Tripsin/EDTA and reseeded in 12 multiwell plates (180 000/well). Cells were grown to 80% confluence and rendered quiescent for 24 h in a medium containing 0.5% FBS. Next, cells were stimulated with LPS (100 ng/mL for 1 h) and exposed to empagliflozin at clinically relevant doses (0.01, 0.1, 1, and 10 μM dissolved in EGM-2 supplemented with 10% FBS; 5 h) before lysis and protein extraction was performed. Stimulation with TNF-α (10 ng/mL) served as a positive control. TF activity was quantified on cell lysate as above-mentioned, and TF and PAI-1 expression were determined by western blotting.

2.7 Western blotting

Protein expression was determined by Western blot analysis as previously described.31,32 Endothelial cells were lysed (50 mmoL/L Tris–
HCl, 100 mmoL/L NaCl, 0.1% Triton X-100, pH 7.4) and total protein concentration was determined according to the manufacturer’s recommendations (Bio-Rad Laboratories AG, Fribourg, Switzerland); 20–30 µg of total protein lysates were separated on an 8 or 10% SDS–PAGE before being transferred to a polyvinylidene fluoride membrane by wet transfer (Bio-Rad Laboratories AG, Fribourg, Switzerland). Membranes were incubated with primary antibodies against TF (ADG4507, clone VIC7; 1:1000, ImmBioMed GmbH & Co KG, Pfungstadt, Germany), PAI-1 (#sc5297, lot #K1308; 1:1000, Santa Cruz), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, #MAB374; 1:20000; Merck Millipore, Billerica, MA, USA) over-night at 4°C on a shaker. Secondary antibodies anti-mouse (#1031-05) and anti-rabbit (#4050-05) were obtained from Southern Biotechnology (Birmingham, AL, USA) and applied for 1 h at room temperature. Densitometric analyses were performed (Amersham Imager 600, General Electric; Healthcare Europe GmbH, Glattbrugg, Switzerland) and protein expression was normalized to GAPDH.

2.8 Human cohort and biochemical analyses

A total of 44 patients with Type 2 diabetes mellitus (T2D) and prevalent ASCVD or at high CV risk were randomly assigned to receive 10 mg empagliflozin or matching placebo daily over a period of 3 months. Randomization was performed in a double-blinded manner with the use of permuted block randomization (block size of 4) to generate the randomization list. Study participants were recruited at the Department of Internal Medicine I at University Hospital Aachen, RWTH Aachen University, Germany. The study has been registered at clinicaltrialsregister.eu (EudraCT number: 2016-000172-19) and its study protocol as well as the baseline (BSL) characteristics of its study population have been described in detail elsewhere.33 Briefly, EDTA blood was drawn at BSL and 3 months FUP, immediately centrifuged, and plasma samples were stored at –80°C before they were transferred to the main institution performing ELISA measurements of PAI-1 and TF (Center for Molecular Cardiology, University of Zurich, Zurich,
respectively). Plasma glucose was assessed by glucometer (Roche, Rotkreuz, Switzerland) and investigators involved in biochemical analyses were fully blinded to treatment allocation. This trial was conducted according to the Declaration of Helsinki, approved by the ethics committee of the University Hospital Aachen (reference number: EK 250/16), and all study participants gave written informed consent.

2.9 Statistical analysis
The random assignment of 9 mice per group was estimated to provide 85% power [at a 95% confidence interval (CI), thereby controlling the type-I error rate α = 0.025] to show equivalence of empagliflozin to vehicle with respect to time-to-arterial occlusion (TTO), using a predefined equivalence margin for the mean between-group difference of 10.5 min, assuming a standard deviation as previously reported.24 Statistical analysis was performed using R Studio 1.3.959 (Vienna, Austria) and GraphPad Prism 8 software. If not stated otherwise, data are presented as mean ± SEM within each group. Between-group differences in means and the corresponding 95% CI thereof are shown in the respective estimation plots, denoted as delta(±).

3. Results
3.1 At basal conditions, arterial thrombus formation is unchanged upon acute empagliflozin administration
To investigate the effect of acute empagliflozin-mediated SGLT-2 inhibition on arterial thrombus formation, time to arterial occlusion (TTO) was analysed in wild-type animals receiving empagliflozin or vehicle 24 and 1 h before photochemical-induced carotid endothelial injury (Figure 1A). Of note, the between-group difference in TTO was within the predefined limit of equivalence (Δ = [10.50; 95% CI, −9.20 to 9.69 min) indicating that empagliflozin does not affect arterial thrombus formation in vivo in non-inflamed mice (Figure 1B and C). Yet, empagliflozin administration significantly reduced plasma glucose levels [delta(−), −1.64, 95% CI, −3.18 to −0.10 mM/L], suggesting that, despite its acute administration, fully exerts its inhibitory effect on SGLT2 in the proximal nephron (Figure 1D). The comparable cyclic flow variations mirror the complex interplay of coagulation cascade activation, platelet aggregation, and fibrinolytic activity (Figure 1E). In line with the above, animals on empagliflozin showed similar thrombus formation dynamics, as no difference in thrombus embolization episode counts was detected (defined as blood flow restoration >0.1 mL/min; 95% CI, −0.60 to 2.31; Figure 1F). Also, BSL heart rate (95% CI, −7.89 to 47.75 bpm) and blood flow (95% CI, −0.09 to 0.09 mL/min) remained unchanged upon empagliflozin treatment (Figure 1G and H, respectively).

3.2 Upon chronic low-grade inflammation, arterial thrombus formation remains unaffected by acute empagliflozin administration
Chronic low-grade inflammation is highly prevalent in diabetics and contributes mechanistically to the high risk for adverse CV events conferred by dysmetabolic states.5,13,15 Thus, we next investigated the effects of empagliflozin treatment on arterial thrombus formation in an established mouse model in which a pro-inflammatory milieu was established by super-low-dose LPS administration, yielding an inflammatory state similarly to diabetic patients at high CV risk.14,15 Applying the same protocol as above, mice were randomly assigned to empagliflozin or vehicle, 24 and 1 h before thrombosis was induced (Figure 2A). Interestingly, the difference in TTO was again within our predefined margin of equivalence (Δ = [10.50; 95% CI, −9.82 to 8.85 min), whereas empagliflozin induced a marked reduction in plasma glucose levels [delta(−), −4.84, 95% CI, −7.95 to −1.74] (Figure 2B). These findings indicate that, despite its pronounced inhibitory effect on SGLT-2 in the proximal nephron, arterial thrombus formation remains unaffected by empagliflozin treatment upon sustained low-grade inflammation (Figure 2C and D). In line with these findings, thrombus embolization counts were not changed by empagliflozin treatment (95% CI, −0.98 to 1.90) (Figure 2E and F), with initial heart rate (95% CI, −51.46 to 25.19 bpm) and blood flow (95% CI, −0.20 to 0.03 mL/min) being comparable between groups (Figure 2G and H, respectively).

3.3 Platelet count, volume, and function remain unaltered upon empagliflozin-mediated SGLT-2 inhibition during sustained low-grade inflammation
Platelet aggregation is a key component of arterial thrombus formation; thus, to deepen mechanistic insights, we next sought to investigate the effect of empagliflozin administration on platelet count, morphology, and function during low-grade inflammatory states. Blood count analysis revealed that platelet numbers (95% CI, −30.27 to 177.30 106/mm3) and volumes (95% CI, −0.10 to 0.20 fL) were not changed upon empagliflozin treatment (Figure 3A and B, respectively). Next, platelet activation status was studied in animals after thrombosis by assessing circulating PF4 levels, a chemokine released upon the reactivity of washed platelets to these mediators by leveraging ex vivo light transmission aggregometry. Of note, empagliflozin did not change the collagen-induced aggregation profile, as demonstrated by the unchanged maximal aggregation (95% CI, −12.07 to 26.94%), comparable rate (slope) of aggregation (95% CI, −10.89 to 29.49%/min) and similar lag phase (95% CI, −5.68 to 0.54 s) (Figure 3D). In concert, a similar aggregation profile was observed by thrombin stimulation, as exemplified by unchanged maximal aggregation (95% CI, −20.23 to 35.77), rate (slope) of aggregation (95% CI, −1.24 to 19.02%/min), and lag phase (−0.63, 95% CI, −3.31 to 2.04 s) upon empagliflozin treatment (Figure 3E).

3.4 SGLT-2 inhibition by empagliflozin does not interfere with the coagulation cascade or fibrinolysis upon low-grade inflammation
Exposure of vascular TF triggers the activation of the coagulation cascade, eventually leading to thrombin and fibrin formation. Consistent with the unperturbed thrombus formation, carotid lysates showed...
Figure 3  Impact of empagliflozin on platelet count, volume, activation, and ex vivo aggregation. (A and B) Platelet count and mean platelet volume in vehicle- vs. empagliflozin-treated animals. (C) PF4 circulating levels after thrombosis in vehicle- vs. empagliflozin-treated animals. (D) Representative traces of ex vivo collagen-induced platelet aggregation. (E–G) maximal aggregation, rate (slope) of aggregation and lag phase during collagen-induced aggregation in the two study groups. (H) Representative traces of ex vivo thrombin-induced platelet aggregation. (I–K) maximal aggregation, rate (slope) of aggregation and lag phase during thrombin-induced aggregation in the two study groups. n = 6 different mice per group (A and B, D–K), n = 8–9 different mice per group (C). Bars and whiskers show means ± SEM, and red rectangles denote difference between means with line-lengths corresponding to the 95% CI. CXCL4 indicates chemokine (C–X–C motif) Ligand 4, and PF4 platelet-factor 4. EMPA indicates empagliflozin-treated (red) and CTRL vehicle-treated mice (blue). A TOST procedure was performed throughout, and limits of the 95% CI are shown.
unchanged TF activity (i.e. reduced activation of factor X at the functional assay) upon empagliflozin-mediated SGLT-2 inhibition (95% CI, 178.10–80.42 pmol/L of protein) (Figure 4A). Accordingly, ELISA quantification of TF in carotid tissues (95% CI, –1478.00 to 229.90 pg/mg of protein) and in plasma (95% CI, –12.93 to 12.57 pg/mL) yielded unaltered levels of the procoagulant (Figure 4B and C, respectively). Endogenous fibrinolysis opposes thrombus formation by degrading fibrin into fibrin degradation products (such as D-dimer) to dissolve the clot. PAI-1 inhibits this process and is the main endogenous regulator of fibrinolytic activity. Of interest, empagliflozin did not impact fibrinolysis, as indicated by unchanged levels of D-dimer (95% CI, –410.80 to 174.70 ng/mL), vascular PAI-1 (95% CI, 252.10–498.90 pg/mg of protein), and plasma PAI-1 (95% CI, –6.66 to 126.90 pg/mL) (Figure 4D–F).

3.5 SGLT-2 inhibition by empagliflozin has no impact on TF activity and PAI-1 expression in inflamed primary HAECs

To test the translational relevance of our findings in vivo, we next sought to assess TF activity and PAI-1 expression in primary HAECs pre-treated with LPS and exposed to increasing doses of empagliflozin (0.01–10 µM) (Figure 5A). In line with the in vivo murine data, empagliflozin did not affect TF activity (LPS and 10 µM empagliflozin vs. LPS; 95% CI, –4.56 to 46.54 pmol/L of protein) in LPS-stimulated HAECs, while TF protein expression was blunted upon empagliflozin exposure (LPS and 10 µM empagliflozin vs. LPS; delta(x̄), –0.55, 95% CI, –0.72 to –0.39) (Figure 5B and C, respectively). Yet, PAI-1 expression remained

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**Figure 4** Effects of empagliflozin on extrinsic coagulation pathway and fibrinolytic system. (A) TF activity in carotid artery lysates of animals treated with empagliflozin or vehicle. (B) Levels of TF in carotid arteries from the two study groups. (C) Levels of TF in plasma samples from the two study groups. (D) D-Dimer concentration in plasma-EDTA of animals after thrombotic occlusion of the right common carotid artery. (E) Levels of PAI-1 in carotid arteries from the two study groups. (F) Plasma levels of PAI-1 in the two study subgroups. n = 8–9 different mice per group. Data are presented as means ± SEM. Note that the red rectangles/lines show the difference between means/95% CI. PAI-1 denotes plasminogen activator inhibitor, and TF tissue factor. EMPA denotes empagliflozin-treated (red) and CTRL vehicle-treated mice (blue). Results are based on TOST procedures, and limits of the 95% CI are shown.
unaffected by empagliﬂozin administration (LPS and 10 μM empagliﬂozin vs. LPS; 95% CI, −0.35 to 0.08) (Figure 5D).

### 3.6 Empagliﬂozin therapy does not impact signatures of fibrinolytic/thrombotic activity in patients with T2DM at high CV risk

To probe whether empagliﬂozin therapy affects key regulatory mechanisms of arterial thrombus formation in humans, plasma TF and PAI-1, proxies of vascular TF and PAI-1, respectively, (see Supplementary material online, Figure S1) were assessed in T2D patients with prevalent ASCVD or high CV risk randomly allocated to 10 mg empagliﬂozin or matching placebo at both BSL and 3 months FUP. Expectedly, in patients on placebo, both plasma TF and PAI-1 levels remained unchanged during FUP (95% CI, −49.09 to 10.36 and −2.06 to 0.70; Figure 6A and B, respectively), with comparable glucose levels at both BSL and FUP (95% CI, −1.93 to 0.54; Figure 6C). Of note, in patients randomized to receive a daily dose of 10 mg empagliﬂozin, both TF and PAI-1 plasma levels, two key markers of thrombotic and fibrinolytic activity, respectively, did not change over time (95% CI, −5.32 to 55.21 and −0.24 to 4.11, respectively; Figure 6D and E, respectively). In contrast, plasma glucose levels declined signiﬁcantly following 3 months empagliﬂozin therapy [delta(\(\bar{x}\)) 1.39, 95% CI, −2.52 to −0.254 mmol/L, Figure 6F].

### 4. Discussion

In the current study, we sought to assess the impact of acute empagliﬂozin-mediated SGLT-2 inhibition on arterial thrombus formation under basal conditions as well as sustained low-grade inﬂammation. Evidence derived from previous randomized controlled trials suggests that SGLT-2 inhibition confers clinical beneﬁts beyond glycaemic control, but whether this involves mechanisms implicated in arterial thrombus formation remained unresolved. In this study, we, therefore, took advantage of an established mouse model in which a sustained low-grade inﬂammatory milieu was established by super-low-dose LPS endotoxaemia, thereby closely mimicking the metabolic milieu of patients at high risk for adverse CV events. Indeed, such super-low-dose LPS administration...
Figure 6  The effects of empagliflozin on proxies of atherothrombotic risk. (A) Plasma TF levels at baseline (BSL) and 3 months follow-up (FUP) of patients assigned to the placebo arm. (B) Longitudinal PAI-1 protein levels in plasma of placebo-treated T2D patients. (C) Plasma glucose levels in patients on placebo. (D) TF plasma levels in patients assigned to empagliflozin treatment. (E) PAI-1 plasma levels in empagliflozin-treated patients. (F) Plasma glucose levels before (BSL) and 3 months after (FUP) empagliflozin treatment was initiated. Bars and error bars show means ± SEM within each group. Rectangles indicate difference between means with line-length corresponding to the 95% CI. ASCVD, atherosclerotic cardiovascular disease; CV, cardiovascular; PAI-1, plasminogen activator inhibitor-1; and TF, tissue factor. A TOST procedure was performed for each comparison, and limits of the 95% CI are shown.
over 4 weeks was shown to markedly increase TNF-α, IL-6, IL-10, and MCP-1, while resulting in higher circulating CD11b+Ly6C<sup>+</sup> pro-inflammatory monocyte counts. These mediators are fundamentally implicated in atherogenesis, link traditional and emerging risk factors to the evolution of atherosclerotic plaques, and participate mechanistically in events occurring within the vascular wall before arterial thrombus formation. By using in vivo, ex vivo, and in vitro approaches, we provide translational evidence demonstrating that acute empagliflozin-mediated SGLT-2 inhibition does not impact experimental arterial thrombus formation, neither during the basal state nor upon sustained low-grade inflammation, reflecting in unchanged plasma TF and PAI-1 levels in patients randomized to a daily dose of 10 mg empagliflozin over 3 months.

Indeed, while empagliflozin induced a reduction of plasma glucose by 1.64 and 4.84 mmoL/L, respectively, in our preclinical models (BSL and super-low-dose LPS treatment, respectively), TTO remained unaffected by empagliflozin treatment at both basal conditions and during low-grade inflammation. The more pronounced decline in plasma glucose in LPS-treated animals is likely a product of perturbed glucose metabolism coupled with diminished insulin sensitivity, as it typically occurs during low-grade inflammatory states, resulting in aggravated urinary glucose excretion upon SGLT-2 inhibition. Indeed, continuous LPS-infusions evoke elevations in blood glucose, insulinemia, and gain in adipose tissue weight to a similar extent as high-fat diets, with CD14 mutant mice (i.e. the main LPS receptor) being mostly resistant to both LPS- and high-fat diet induced dysmetabolism. Whether such phenotypic changes also occur at super-low-doses of LPS, as it was used in the current study, needs to be addressed by future studies.

The unaltered thrombus formation kinetics in vivo was recapitulated by ex vivo platelet aggregometry, in which neither collagen- nor thrombin-induced platelet aggregation was altered by empagliflozin administration. This is in line with a previously published abstract, where pigs were randomly assigned to empagliflozin or placebo, showing that neither thrombus kinetics (assessed by rotational thromboelastography) nor ADP/collagen-induced platelet aggregation were altered by empagliflozin treatment. During aggregation, platelets are progressively activated resulting in the release of PF4, which, through its neutralizing activity of heparin-like molecules, accelerates coagulation cascade activity. A former study involving 20 patients with stable coronary artery disease and Type 2 diabetes reported slightly attenuated P2Y<sub>12</sub> Platelet reactivity 10 days after daily empagliflozin treatment was initiated, yet these findings are limited by a relatively small sample size, concomitant dual ant platelet therapy, unavailability of additional readouts, and predominance of South Asian ethnicity. In contrast, we found that PF4 levels are unchanged upon SGLT-2 inhibition, which is coherent to our ex vivo study on platelet aggregation using two independent triggers.

Following platelet aggregation and activation of the extrinsic coagulation cascade by TF, activated platelets adhere to leukocytes (mainly via the interaction of P-selectin and P-selectin glycoprotein ligand-1) to eventually form mixed thrombus aggregates that are further stabilized by both thrombin and fibrin. Notably, TF activity was similar in both carotid lysates obtained from empagliflozin- vs. vehicle-treated animals and HAE Cs pre-treated with LPS with and without high-dose empagliflozin, suggesting that the extrinsic coagulation cascade remains unaffected by acute SGLT-2 inhibition. The endogenous fibrinolytic system antagonizes stable thrombus formation, resulting in the systemic release of fibrin degradation products, such as D-dimer, whose levels were similar between the two groups, collectively suggesting that both the extrinsic coagulation cascade and the fibrinolytic system operate unaffectedly by empagliflozin administration.

The pronounced correlation of plasma TF and PAI-1 with vascular TF and PAI-1 (see Supplementary material online, Figure S1), their mechanistic implications in atherothrombosis and their postulated effects on atherothrombotic risk, prompted us to assess whether empagliflozin therapy alters TF and PAI-1 plasma levels in patients at low-grade inflammatory state. To that end, T2D patients with prevalent ASCVD or at high CV risk were randomized to receive a daily dose of 10 mg empagliflozin or matching placebo, and proxies of atherothrombotic risk, namely TF and PAI-1 antigen levels, were assessed at both BSL and 3 months FUP. Interestingly, while both TF and PAI-1 plasma levels remained unchanged over time, a significant reduction in plasma glucose could be observed. Considering that blood-derived TF links CV risk factors to atherothrombosis, and PAI-1 antigen levels confer heightened risk to atherothrombotic events, these observations add an additional layer of human evidence that empagliflozin-mediated SGLT-2 inhibition does not alter key players of atherothrombosis. Of note, a previous study reported declined PAI-1 plasma levels in patients on empagliflozin. Given the well-described interaction of body weight changes and PAI-1 levels, a phenomenon similarly observed in the aforementioned study, we anticipate that these intriguing findings are mainly due to body weight reduction rather than empagliflozin-mediated SGLT-2 inhibition, as no changes in body weight were observed in our study, as reported previously.

But what is the action by which SGLT-2 inhibition improves clinical outcomes in patients at high CV risk beyond glycaemic control? Besides glucose lowering, natriuresis and alterations in tissue sodium handling likely contribute to cardiorenal benefits and improved outcomes of patients with ASCVD receiving the active drug. Indeed, changes in tissue sodium handling coupled with enhanced natriuresis lead to reductions in plasma volume and thus cardiac preload, attenuated systemic blood pressure, reduced vascular stiffness, and diminished albuminuria, which collectively preserves renal function and reduces heart failure progression. To avoid effects on multiple organ-systems inherent to long-period treatments, we therefore administered empagliflozin acutely, a time lapse sufficient to observe the antihyperglycemic effects, even under BSL conditions. Although further studies are warranted to deepen our mechanistic understanding of the pathways by which SGLT-2 inhibition provides clinical benefits, it is now widely accepted that the glucose-lowering action of SGLT-2 inhibitors only represents one aspect of their cardiorenal benefits.

Our study has certain limitations inherent to its design which deserve discussion. First, only acute effects of empagliflozin treatment were assessed in the current study. Although this sufficed to exert its glucose-lowering effects, we cannot exclude that long-term administration would interfere with arterial thrombus formation. Yet, as a former study could establish alterations of (sub-)cellular functions in ZDF obese rats could interfere with arterial thrombus formation. Yet, as a former study could establish alterations of (sub-)cellular functions in ZDF obese rats already after 30 min upon a single-dose of 0.25 mg/kg empagliflozin, we anticipate that direct effects on arterial thrombosis would likely occur within 24 h, if present. Nevertheless, long-term treatment was consistently shown to have multifaceted effects on haemodynamics and rheologic factors, which might indirectly impact arterial thrombus formation; however, this represents an interesting field of research that certainly warrants further investigations. Second, the in vivo model employed is based on a previously reported murine model of low-grade inflammation in the context of atherosclerosis, rather than heart failure. Indeed, although the indication for empagliflozin has been expanded to the heart failure spectrum recently, most patients enrolled in contemporary trials had established ASCVD with up to 50% being afflicted by diabetes. Hence, a model displaying a phenotype shared by patients
enrolled in previous trials was used in the current study. Third, although murine platelets share many functional properties of human platelets, they are smaller in size, more abundant in granule heterogeneity and differ in protein expression profile, as reviewed in detail previously, thus the ex vivo findings presented herein may be limited by these phenotypic differences. In addition, while carotid tissue lysates obtained from empagliflozin- vs. vehicle-treated animals showed unchanged TF protein expression, LPS-pre-treated HAECs showed intriguingly blunted TF expression with increasing empagliflozin dosages. Given, however, that TF activity remained unaffected by empagliflozin administration, we argue for a finding with no physiological relevance. Additionally, our photochemical thrombosis model allows for the study of endothelial-specific rather than a transmural damage-induced arterial thrombosis, the former representing a much more translational setting; yet the use of other thrombosis models may have yielded distinct results. Further, ex vivo platelet aggregation was studied using washed platelets in the absence of calcium, the prolonged rise in intracellular calcium may have led to diminished platelet reactivity and as such mitigated platelet aggregation. In aggregate, this study demonstrates for the first time that acute SGLT-2 inhibition by empagliflozin does not impact experimental arterial thrombus formation in non-diabetic mice, neither at the basal state nor during sustained low-grade inflammation. Data obtained in mice, human cells, and ASCVD patients suggest that empagliflozin administration likely improves outcomes independently of pathways involved in arterial thrombus formation and open an exciting avenue for future in-depth studies to disentangle the molecular basis of its pleiotropic effects.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Authors’ contribution
LL, S.K., F.P., and G.G.C. conceived the project and the experimental set-up; LL, S.K., Y.M.P., N.R.B., S.M., F.A.W. performed animal and in vitro experiments; N.M., M.L., and N.-U.K.H. enrolled the clinical cohort and provided clinical samples; LL, S.K., and G.G.C. wrote the manuscript; all authors provided critical input, critically reviewed the manuscript, and contributed to discussion and interpretation of results.

Conflict of interest: LL and G.G.C. are coinventors on the International Patent (WO/2020/226993) filed in April 2020 and relating to the use of antibodies, which specifically bind IL-1α to reduce various sequelae of ischaemia–reperfusion injury to the central nervous system. G.G.C. is a consultant to Sovida solutions limited. LL has received speaker fees outside of this work from Daichi-Sankyo. T.F.L. has received honoraria and educational grants from Boehringer Ingelheim Switzerland and Ingelheim, FRG, respectively. N.M. has received support for clinical trial leadership from Boehringer Ingelheim, served as a consultant to Boehringer Ingelheim, Merck, AstraZeneca, BMS, received grant support from Boehringer Ingelheim, Merck, and served as a speaker for Boehringer Ingelheim, Merck, Novo Nordisk, Lilly, BMS, and AstraZeneca. M.L. received grants and personal fees from Boehringer Ingelheim, MSD, Novo Nordisk and personal fees from Amgen, Sanofi, Astra Zeneca, Bayer, Lilly, Daiichi Sankyo, Novartis, Amarin. The other authors report no conflict of interest.

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Data availability
The original data underlying this article will be shared upon reasonable request to the corresponding author.

References

L. Liberale et al.
Translational perspective

Sodium–glucose co-transporter 2 (SGLT-2) inhibition improves outcomes in patients with atherosclerotic cardiovascular (CV) disease (ASCVD) independently of its glucose-lowering effects. Low-grade inflammation couples dysmetabolic states to insulin resistance and ASCVD, a frequent cause of heart failure. Employing an established murine model of low-grade inflammation, we show that acute empagliflozin administration affects arterial thrombus formation equivalent to vehicle. These findings are corroborated by ex vivo and in vitro experiments on murine platelets and primary human endothelial cells. Unchanged proxies of the fibro-coagulative state [i.e. plasma tissue factor (TF)/PAI-1] in patients at high risk for ASCVD and its dreadful sequelae randomly assigned to empagliflozin therapy suggest that SGLT-2 inhibition improves outcomes via mechanisms independent from arterial thrombosis.