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Empagliflozin decreases ageing-associated arterial stiffening and vascular fibrosis under normoglycemic conditions

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ABSTRACT

Arterial stiffness is a hallmark of vascular ageing and results in increased blood flow pulsatility to the periphery, damaging end-organs such as the heart, kidneys and brain. Treating or "reversing" arterial stiffness has therefore become a central target in the field of vascular ageing. SGLT2 inhibitors, initially developed in the context of type 2 diabetes mellitus, have become a cornerstone of heart failure treatment. Additionally, effects on the vasculature have been reported. Here, we demonstrate that treatment with the SGLT2 inhibitor empagliflozin (7 weeks, 15 mg/kg/day) decreased ageing-induced arterial stiffness of the aorta in old mice with normal blood glucose levels. However, no universal mechanism was identified. While empagliflozin reduced the ageing-associated increase in collagen type I in the medial layer of the abdominal infrarenal aorta and decreased medial TGF-β deposition, this was not observed in the thoracic descending aorta. Moreover, empagliflozin was not able to prevent elastin fragmentation. In conclusion, empagliflozin decreased arterial stiffness in aged mice, indicating that SGLT2 inhibition could be a valuable strategy in mitigating vascular ageing. Further research is warranted to unravel the underlying, possibly region-specific, mechanisms.

1. Introduction

Arterial stiffness, a hallmark of vascular ageing, increases blood flow pulsatility and therefore causes damage to low impedance end-organs such as the brain, the heart and kidneys [1–3]. Both functional and structural changes occur during vascular ageing that synergistically contribute to the development of blood vessel stiffening. Functional changes relate to the altered contractile state of vascular smooth muscle cells (VSMCs). Altered VSMC function is caused by, in part, adaptations in their response to vasoconstrictors, decreased nitric oxide (NO) bioavailability due to endothelial dysfunction and modified cell-matrix adhesion [1,4,5]. Extracellular matrix (ECM) remodelling is also part of vascular ageing and contributes to progressive stiffening of the vascular wall. Such structural changes include, among others, elastin fragmentation, medial calcification and collagen deposition [6–8]. While the ageing-related structural and functional changes are mentioned here as separate events, a complex crosstalk between VSMCs and the ECM exists that could further drive the progressive impairment of vascular homeostasis [9,10].

Empagliflozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor, is an approved antihyperglycemic agent used in the treatment of type 2 diabetes mellitus (T2DM) [11,12]. SGLT2 co-transporters are located at the proximal tubules of the kidney and reabsorb filtered glucose. Therefore, inhibition of SGLT2 reduces glucose reabsorption and lowers (excess) plasma glucose [13]. This warrants the use of agents such as empagliflozin in the treatment of T2DM. Interestingly, SGLT2 inhibitors also have beneficial cardiovascular effects independent of their glucose-lowering effect [14–16]. For example, both empagliflozin and dapagliflozin have been shown to lower the risk of mortality in heart failure, independent of the presence of T2DM [17,18]. Moreover, chronic dapagliflozin treatment attenuates vascular endothelial dysfunction in high fat diet fed ApoE$^{-/-}$ mice, with normal glucose levels [19]. In addition, empagliflozin has been shown to restore inflammation-induced microvascular endothelial dysfunction [20]. Recently, it has been demonstrated that SGLT2 is expressed in vascular cells as well, which highlights the possibility for direct effects of SGLT2 inhibitors on the vasculature [21]. Indeed, both dapagliflozin and empagliflozin have been shown to induce vasodilation by stimulating smooth muscle cell

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voltage-gated K^+ channels [22,23]. These studies demonstrate that SGLT2 inhibitors have protective effects on the vasculature, independent of their glucose-lowering effect and therefore highlighting their pleiotropic properties.

The present study aimed to address whether empagliflozin can attenuate ageing-induced stiffening of the aorta, in the absence of hyperglycemia. We assessed *ex vivo* aortic stiffness of central blood vessels in both young and old mice after chronic treatment (7 weeks) with empagliflozin (15 mg/kg/day). An in-house developed set-up to study arterial compliance (ROTSAC) was used to assess arterial stiffness *ex vivo,* while vascular reactivity was measured in conventional organ baths. Finally, we evaluated whether ageing affected ECM composition of the aortic wall on a histological level and whether empagliflozin can alter such age-related changes.

2. Methods

2.1. Chemical compounds

Empagliflozin was purchased from MedChemExpress (Bio-connect, HY-15409, The Netherlands). Phenylephrine, L-NG-Nitroarginine methyl ester (L-NAME), acetylcholine (ACh), diethylamine Nitric Oxide (DEANO) and diltiazem were purchased from Sigma-Aldrich (Belgium). Levcromakalim was purchased from TOCRIS (United Kingdom).

2.2. Mice and tissue preparation

Female C57BL/6J mice (Charles River, France), either 4 months (young cohort) or 26 months old (aged cohort), were used for all experiments. All animals were housed in the animal facility of the University of Antwerp in standard cages with 12 h–12 h light-dark cycles and had free access to regular chow and tap water. The animals were anaesthetised with sodium pentobarbital (Sanofi, Belgium), 75 mg/kg (i.p.) and subsequently euthanized by perforating the diaphragm. The heart, liver, lungs, spleen and kidneys were collected and weighted. Furthermore, the ascending, descending and infrarenal aorta were carefully removed and stripped of all adherent tissue. The isolated blood vessels were then cut into segments of 2 mm length. The segments were immersed in Krebs Ringer (KR) solution (37 °C, 95% O_2/5% CO_2, pH 7.4) containing (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, CaEDTA 0.025 and glucose 11.1. All animal experiments were approved by the Ethical Committee of the University of Antwerp (ECD n° 2022/43) and were conducted in accordance with the EU Directive 2010/63/EU.

2.3. Empagliflozin treatment

Young (control: $n = 6$; empagliflozin: n = 6) and aged (control: $n = 7$; empagliflozin: n = 7) mice were treated with empagliflozin (15 mg/kg/day) for 7 weeks by dissolving the compound directly in the drinking water (200 μM empagliflozin). Drinking bottles were changed twice a week. Because no vehicle was used to dissolve empagliflozin, age-matched controls received normal drinking water.

2.4. Blood glucose measurements

Blood glucose levels were measured after 7 weeks of treatment. In brief, a small sample of blood was taken from the tail vein of restrained mice in the morning (fed glucose levels) and again 5 h after food restriction (fasted glucose levels). Blood glucose measurements were performed with a hand-held glucometer (OneTouch Verio®glucometer, range, 20–600 mg/dL; Lifescan, Milpitas, CA, USA).

2.5. Measuring ex vivo aortic stiffness

The *ex vivo* stiffness of aortic vessels was determined *via* "Rodent Oscillatory Tension Set-up to Study Arterial Compliance" (ROTSAC) measurements as previously described [24]. In brief, aortic segments were mounted between two parallel wire hooks in 10 mL organ baths. Force and displacement of the upper hooks were controlled and measured with a force length transducer. The segments were continuously stretched between alternating preloads, corresponding to a "diastolic" and "systolic" transmural pressure at a frequency of 10 Hz to mimic the physiological heart rate in mice (600 bpm). The Laplace relationship was used to calculate the transmural pressure. At any given pressure, calibration of the upper hook allowed the calculation of the vessel diameter (both systolic and diastolic diameter) and the Peterson pressure-strain modulus of elasticity (Ep). The Ep was calculated as follows: $Ep = D_0 . \Delta P/\Delta D$, with ΔP = difference in pressure (kept constant at 40 mmHg), D_0 = "diastolic" diameter and ΔD = the change in diameter between "diastolic" and "systolic" pressure. The ROTSAC protocol included the evaluation of arterial stiffness (Ep) at different pressures (*i.e.*, 40–80 until 220–260 mmHg with 20 mmHg intervals), under a physiological/active state (Krebs-Ringer solution), as well as the contribution of the passive components by adding a high concentration (10 μM) of the nitric oxide donor Diethylamine Nitric Oxide (DEANO).

2.6. Measuring vascular reactivity

Thoracic aortic segments were mounted at a constant preload of 20 mN in organ baths to measure isometric force development. For optimal stabilization and to minimize the effects of NO in a time dependent manner, all experiments commenced 60 min after perforation of the diaphragm. VSMC contraction was evaluated by adding cumulative concentrations of phenylephrine (3 nM-3 μM), an α1-adrenergic receptor agonist. Subsequently, endothelial-dependent relaxations were evaluated by adding cumulative concentrations of ACh (3 nM-10 μM), a muscarinic receptor agonist. Levcromakalim, a K_{ATP} channel opener, was administered to evaluate the contribution of relaxation due to K^+ efflux. Diltiazem, an inhibitor of calcium ion influx, was administered to evaluate the contribution of voltage-gated calcium channels (VGCC) during contraction. To evaluate differences in resting membrane potential, a concentration response of the membrane depolarizer potassium (10 mM–50 mM), was measured. To negate the influence of NO, a non-selective NO synthase inhibitor (L-NAME, 300 μM), was administered.

2.7. Histology

Ascending, descending and infrarenal aortic segments were fixed for 24 h in 4% formaldehyde solution (BDH Prolabo, VWR, Belgium), and subsequently dehydrated in 60% isopropanol, followed by paraffin-embedding. Elastin was visualized by staining cross-sections with Orcein. Afterwards, images were analysed in Fiji/ImageJ and elastin breaks were manually counted. Medial wall thickness was also determined on orcein-stained samples. Collagen composition of the media was evaluated by immunohistochemical (IHC) staining with rabbit polyclonal anti-collagen I (Abcam, ab21286, 1/500 dilution) and rabbit anti-collagen III (Abcam, ab7778, 1/300 dilution). The amount of TGF-β in the medial layer of the aorta was evaluated by IHC using rabbit anti-TGF beta 1 (Abcam, ab25121, 1/200 dilution). All images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope. Image analysis was conducted in Fiji/ImageJ. Collagen content and TGF-β amount were quantified as "percentage area positivity" in the region of interest (*i.e.*, the medial layer). Of note, histology was performed on samples after biomechanical testing.

2.8. In vitro collagen assay

Murine primary vascular smooth muscle cells were isolated from the aorta of 4-week-old female C57Bl6/J mice as previously described [25]. The heart was flushed with Hank's Balanced Salt Solution (HBSS) to

Table 1
General characteristics of young and aged C57Bl6/J mice treated with or without 15 mg/kg/day empagliflozin (EMPA) for 7 weeks.

	Young (4 months)		Aged (26 months)		*p*-value (Age effect)
	Control	EMPA	Control	EMPA	
Body Weight (g)	31.2 ± 0.4	28.8 ± 0.5	35.0 ± 1.7	34.4 ± 1.3	***
Heart Weight/BW (mg/g)	4.4 ± 0.2	5.1 ± 0.3	4.8 ± 0.3	4.5 ± 0.1	ns
Liver Weight/BW (mg/g)	50.7 ± 2.3	52.2 ± 2.0	45.7 ± 1.8	49.2 ± 1.8	ns
Spleen Weight/BW (mg/g)	2.7 ± 0.5	2.7 ± 0.2	7.1 ± 2.1	3.2 ± 0.4$^{\#}$	*
Kidney Weight/BW (mg/g)	11.4 ± 0.7	12.5 ± 0.2	13.1 ± 0.7	14.5 ± 0.6	**
Lung Weight/BW (mg/g)	4.3 ± 0.5	5.1 ± 0.1	5.6 ± 0.7	5.2 ± 0.2	ns
Blood Glucose (mg/dL)					
Fed	151 ± 7	149 ± 3	156 ± 6	155 ± 5	ns
Fasted	124 ± 11	114 ± 4	131 ± 8	137 ± 9	ns

Data are represented as mean ± SEM. Statistical analysis using Two-way ANOVA with a Sidak *post hoc* test for multiple comparisons (comparisons were made between the EMPA and control group of either young or aged mice). $n = 6$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Multiple comparisons: $^{\#}p < 0.05$. **BW** = Body Weight.

remove blood from the aorta. Next, the aorta was incubated for 15 min at 37 °C in HBSS supplemented with 1 mg/mL collagenase (Worthington, type II, LS004174), 1 mg/mL soybean trypsin inhibitor (Worthington, LS003570) and 0.744 units/mL elastase (Worthington, LS002279), in 95%/5% O_2/CO_2. Next, the adventitia was carefully stripped off, after which the aorta was cut into smaller segments which were incubated for 75 min in fresh enzyme solution (as described above) at 37 °C in 95%/5% O_2/CO_2. Cells were collected after centrifugation of the enzyme solution and washed/resuspended in DMEM-F12 (Gibco Life Sciences, 11320-074), supplemented with 20% FBS. Cells from passages 3, 4, 8 and 9 were used in this experiment. Cells were seeded in 8-well cell culture slides (Life Sciences, 354,118) and left undisturbed overnight to allow attachment to the surface of the slide. After 18 h starvation (in DMEM-F12 supplemented with 2% FBS), cells were treated with vehicle, 10 ng/mL TGF-β (BioLegend, 763102), empagliflozin (50 μM) or a combination of TGF-β and empagliflozin for 48 h. After the treatment period, cells were fixed in Bouin's fixative for one hour after which a Sirius red staining was performed to visualize collagen (haematoxylin counterstained). Images were acquired using an Olympus BX40 microscope. Image analysis was performed in Fiji (ImageJ). Collagen content was analysed as: ($Area_{collagen}$/number of haematoxylin positive nuclei) per image.

2.9. Statistics

All results are expressed as mean ± standard error of the mean (SEM) with n representing the number of mice/experimental repeats. Statistical analyses were performed in GraphPad Prism 9.2. Statistical tests are mentioned in the figure and/or table legends. Significance was accepted at $p < 0.05$.

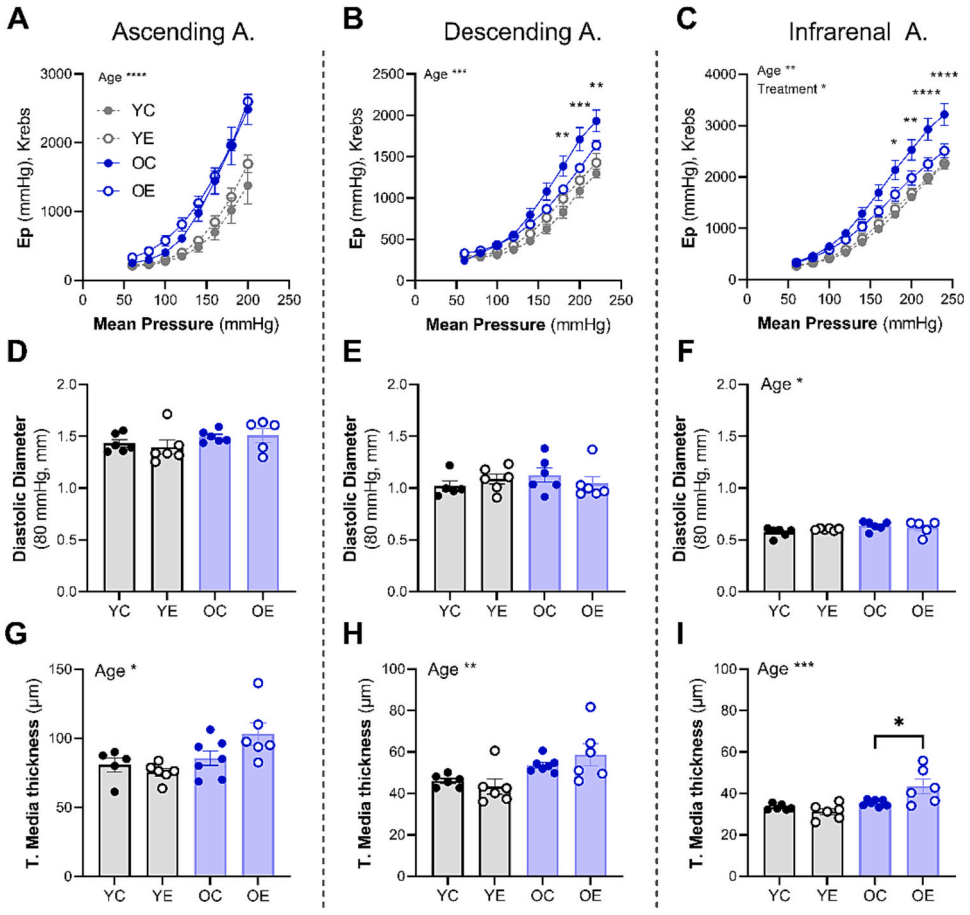

Fig. 1. Empagliflozin decreases *ex vivo* aortic stiffness in old mice. Young and old mice were treated with empagliflozin (15 mg/kg/day) for 7 weeks. Afterwards, the central aorta was collected and subjected to *ex vivo* biomechanical testing. The stiffness of the (A) thoracic ascending aorta (TAA), (B) thoracic descending aorta (TDA) and the (C) abdominal infrarenal aorta (AIA) significantly increased with ageing ($n = 6$ per group). Interestingly, 7 weeks of empagliflozin treatment significantly decreased aortic stiffness in both the TDA and the AIA, but not in the TAA, especially at high *ex vivo* pressures. Although there was no change in diameter (measured at 80 mmHg) between the groups in the (D) TAA and (E) TDA, (F) AIA segments from old mice had a significantly ($p < 0.05$) larger diameter than their control littermates ($n = 5$–6 per group). Medial wall thickness significantly increased with ageing in the (G) TAA (n = 5–7 per group), (H) TDA ($n = 6$–7 per group) and (I) AIA (n = 6–7 per group). Surprisingly, empagliflozin-treated old mice had a significantly thicker medial wall compared to their aged, control counterparts. Statistical analyses: (A-C) Three-way ANOVA with Sidak post-hoc test for multiple comparisons; (D-I) Two-way ANOVA with Sidak post-hoc test for multiple comparisons $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. **YC** = Young mice, control; **YE** = Young mice + empagliflozin: **OC** = Old mice, control: **OE** = Old mice + empagliflozin.

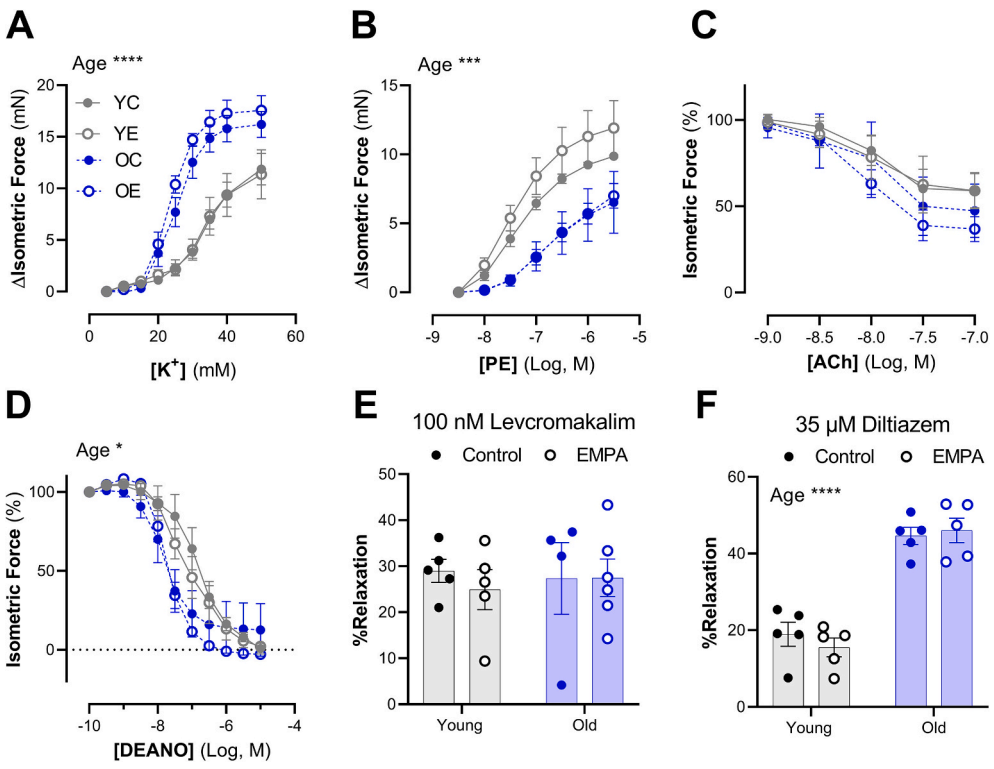

Fig. 2. Empagliflozin does not alter ageing-induced changes in vascular reactivity. Vascular reactivity (*i.e.*, contraction and relaxation) was assessed on thoracic descending aortic tissue from young and old mice, treated with or without empagliflozin (15 mg/kg/day) for 7 weeks. (A) Receptor-independent contraction was assessed *via* a concentration-response stimulation with potassium chloride. Although there was an effect of ageing, empagliflozin did not attenuate the heightened sensitivity of old aortic segments to [K^+]. (B) Receptor-mediated contraction was assessed *via* concentration-response stimulation with phenylephrine (α_1-adrenoceptor). Similarly, ageing decreased the development of isometric force in response to phenylephrine, but empagliflozin did not affect this lowered contractile response. (C) A concentration-response stimulation with acetylcholine was used to assess endothelium-dependent vasorelaxation. However, no differences were observed between the groups. (D) Endothelium-independent vasorelaxation was studied by a concentration-response stimulation with DEANO. Whereas aged mice had a significantly altered response to DEANO, empagliflozin did not alter this response. (E) Levcromakalim (LEV), a K_{ATP} channel opener, induces relaxation on pre-contracted segments. However, no differences were observed in relaxation between the different groups after maximum stimulation of K_{ATP} channels with 100 nM LEV. (F) Diltiazem, a voltage-gated calcium channel inhibitor, induces relaxation on pre-contracted segments. The response to maximum VGCC inhibition with diltiazem was significantly increased in old mice. Empagliflozin did not alter this intensified response to diltiazem. Statistical analyses: Two-way ANOVA with Sidak *post hoc* test for multiple comparisons. A-D; $n = 6$. E; $n = 5$ (young), $n = 4$–6 (old). F; n = 5 per group. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ **PE** = phenylephrine; **ACh** = acetylcholine; **EMPA** = empagliflozin; **YC** = Young mice, control; **YE** = Young mice + empagliflozin: **OC** = Old mice, control: **OE** = Old mice + empagliflozin.

3. Results

3.1. General characteristics of spontaneously aged female C57Bl6/J mice

Whereas body weight increased with ageing, empagliflozin did not alter body weight in young or old mice (Table 1). Neither natural ageing nor empagliflozin altered the heart weight over body weight ratio (HW/BW), an indicator of cardiac hypertrophy. Interestingly, whereas aged mice had a significantly higher spleen weight over body weight ratio, empagliflozin significantly decreased spleen weight in aged mice. Furthermore, the kidney weight over body weight ratio was significantly increased in aged mice, but empagliflozin did not alter this effect. There were no differences in either liver weight or lung weight (normalized to body weight) between the different groups. Finally, fed and fasted plasma glucose concentrations did not differ between old and young mice.

3.2. Empagliflozin decreases ageing-induced arterial stiffness

Ageing significantly increased arterial stiffness in the thoracic ascending aorta (TAA), thoracic descending aorta (TDA) and the abdominal infrarenal aorta (AIA) (Fig. 1, A-C). *Ex vivo* Ep, a measure for stiffness, increased with increasing pressure as previously reported [26]. However, treating old mice (26 months) with empagliflozin (15 mg/kg/day) significantly affected the *ex vivo* biomechanical properties of the aorta. Both the TDA and AIA of the OE group (old mice + empagliflozin) showed a significantly decreased pressure-dependency of E_P compared to old control mice ("OC"), especially at high *ex vivo* pressures. This effect was not observed in the TAA. Furthermore, there were no differences in biomechanical properties between TDA and AIA segments from OE compared to both YC and YE ("Young Control" and "Young Empagliflozin-treated mice"). There were also no differences in aortic stiffness between YC and YE. Next, there were no differences in the diastolic diameter (measured at 80 mmHg) of TAA and TDA aortic segments between the different groups (Fig. 1, D, E). However, ageing significantly increased the diameter of AIA segments at 80 mmHg (Fig. 1, F). Lastly, the thickness of the medial wall increased with ageing (Fig. 1, G-I). Surprisingly, empagliflozin-treated old mice had a significantly thicker medial wall in the AIA compared to their aged, control counterparts. (Fig. 1, I).

3.3. Empagliflozin does not alter age-related changes in ex vivo vascular reactivity

Whereas aged mice had a significantly higher ($p < 0.001$) contractile response to [K^+], aged mice had a significantly decreased contractile response to phenylephrine (Fig. 2A-B). Furthermore, endothelial cell dysfunction was not observed in old mice, as demonstrated by equal responses to ACh (Fig. 2, C). Alternatively, endothelium-independent relaxations with DEANO were significantly left-shifted in old mice (Fig. 2, D). Although treating old mice with empagliflozin decreased aortic stiffness, it did not affect the age-related changes in vascular reactivity. Empagliflozin did not restore the ageing-induced changes in both non-receptor-mediated contraction and α_1-adrenoceptor stimulated contraction with potassium chloride and phenylephrine, respectively (Fig. 2, A, B). Furthermore, empagliflozin treatment did not lead to changes in endothelium-dependent and endothelium-independent relaxations induced by acetylcholine and DEANO (Fig. 2 C, D).

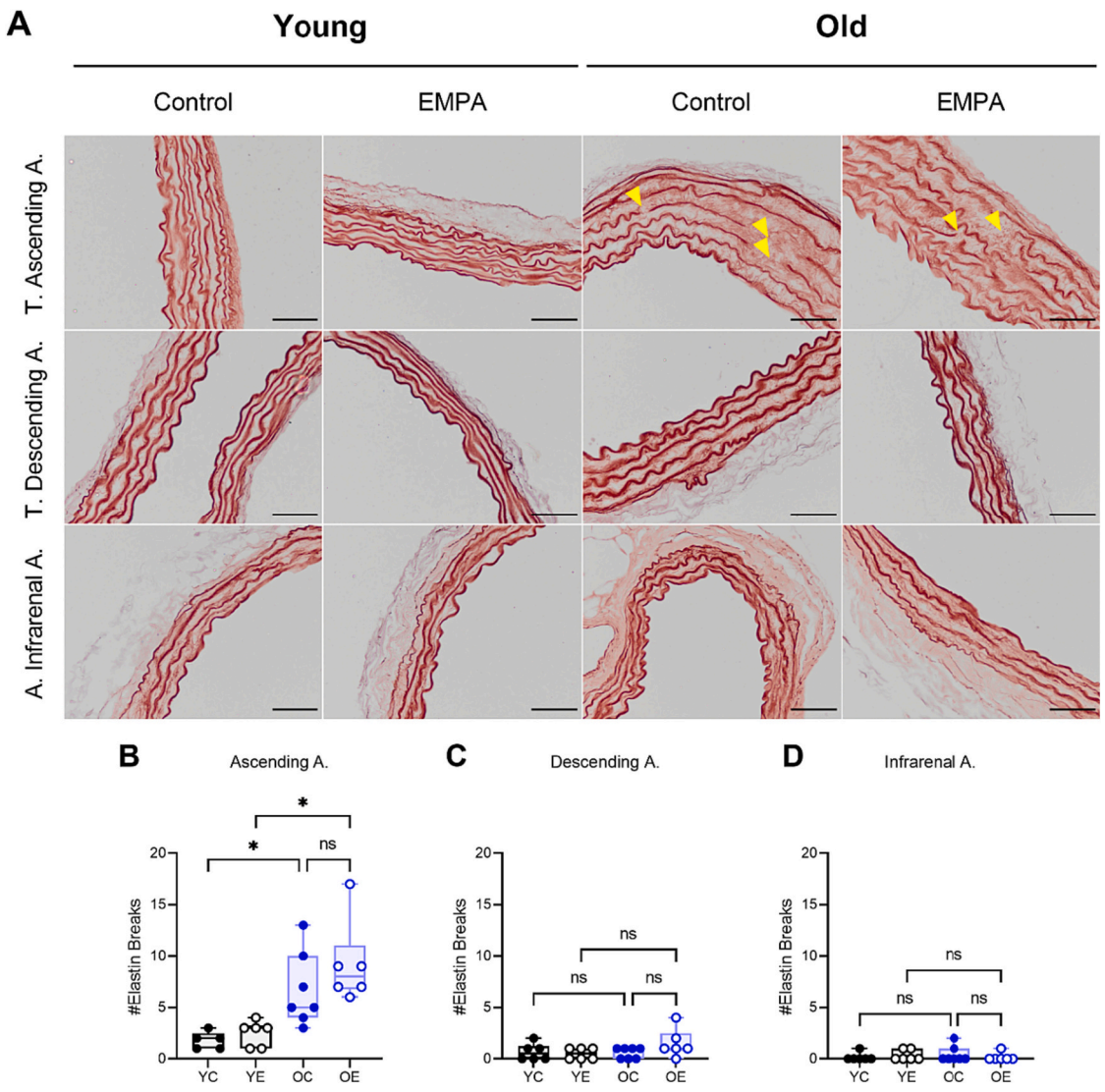

Fig. 3. Empagliflozin does not prevent and/or restore ageing-induced elastin fragmentation in the thoracic ascending aorta. An Orcein staining was performed to visualize elastin fibres in aortic tissue. (A) representative images of orcein-stained aortic cross-sections from old and young mice, treated with or without empagliflozin (15 mg/kg/day). Yellow arrows indicate elastin breaks. Scale bar = 50 μm. (B) Quantification of the amount elastin breaks revealed a significantly increased amount of elastin fragmentation in the thoracic ascending aorta (TAA) from old mice. Empagliflozin treatment did not decrease/increase elastin fragmentation in the TAA. (C, D) No elastin fragmentation was observed in the thoracic descending aorta and the abdominal infrarenal aorta. $n = 5–7$. Statistical analysis was performed by using a Kruskal-Wallis test with Dunn's *post hoc* test for multiple comparisons. TAA; n = 5–7. TDA; $n = 6–7$. AIA; n = 6–7. * = $p < 0.05$; ** = $p < 0.01$. **T. Ascending A.** = Thoracic Ascending Aorta; **T. Descending A.** = Thoracic Descending Aorta; **A. Infrarenal A.** = Abdominal Infrarenal Aorta: **YC** = Young mice, control; **YE** = Young mice + empagliflozin: **OC** = Old mice, control: **OE** = Old mice + empagliflozin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Neither ageing nor empagliflozin affected contraction-inhibition by levcromakalim, a potassium-channel opening vasodilator, on pre-contracted aortic segments with 2 μM PE (Fig. 2, E). Lastly, ageing significantly intensified the contraction-inhibition with diltiazem (35 μM) (Fig. 2, F). However, empagliflozin did not alter this higher vaso-dilatory response.

3.4. *Ageing induces elastin fragmentation, which is not restored by empagliflozin*

An orcein stain was performed on aortic samples to visualize elastin (Fig. 3, A). There was a significantly increased number of elastin breaks in the TAA of aged mice compared to young mice, but not in the TDA or AIA (Fig. 3, B–D). However, there was no difference in the number of elastin breaks in the TAA of old mice treated with empagliflozin compared to old control mice (Fig. 3, B). Empagliflozin did not attenuate or prevent elastin fragmentation.

3.5. *Empagliflozin decreases collagen type I in the infrarenal aorta, but not in the thoracic descending aorta*

Immunohistochemical stains for collagen type I and collagen type III were performed on aortic samples from old and young mice, treated with or without empagliflozin (Fig. 4, A). There was a significantly higher amount of collagen type I deposition in the tunica media of the AIA in old mice compared to young mice (Fig. 4, B). Interestingly, seven weeks of empagliflozin treatment in old mice significantly decreased collagen type I deposition in the tunica media of the AIA, compared to their, old, control littermates (Fig. 4, B). There were no age- or empagliflozin-related changes in medial collagen type I content in the TDA (Fig. 4, C). Interestingly, whereas ageing significantly decreased medial collagen type III in the AIA, an increase in collagen type III was

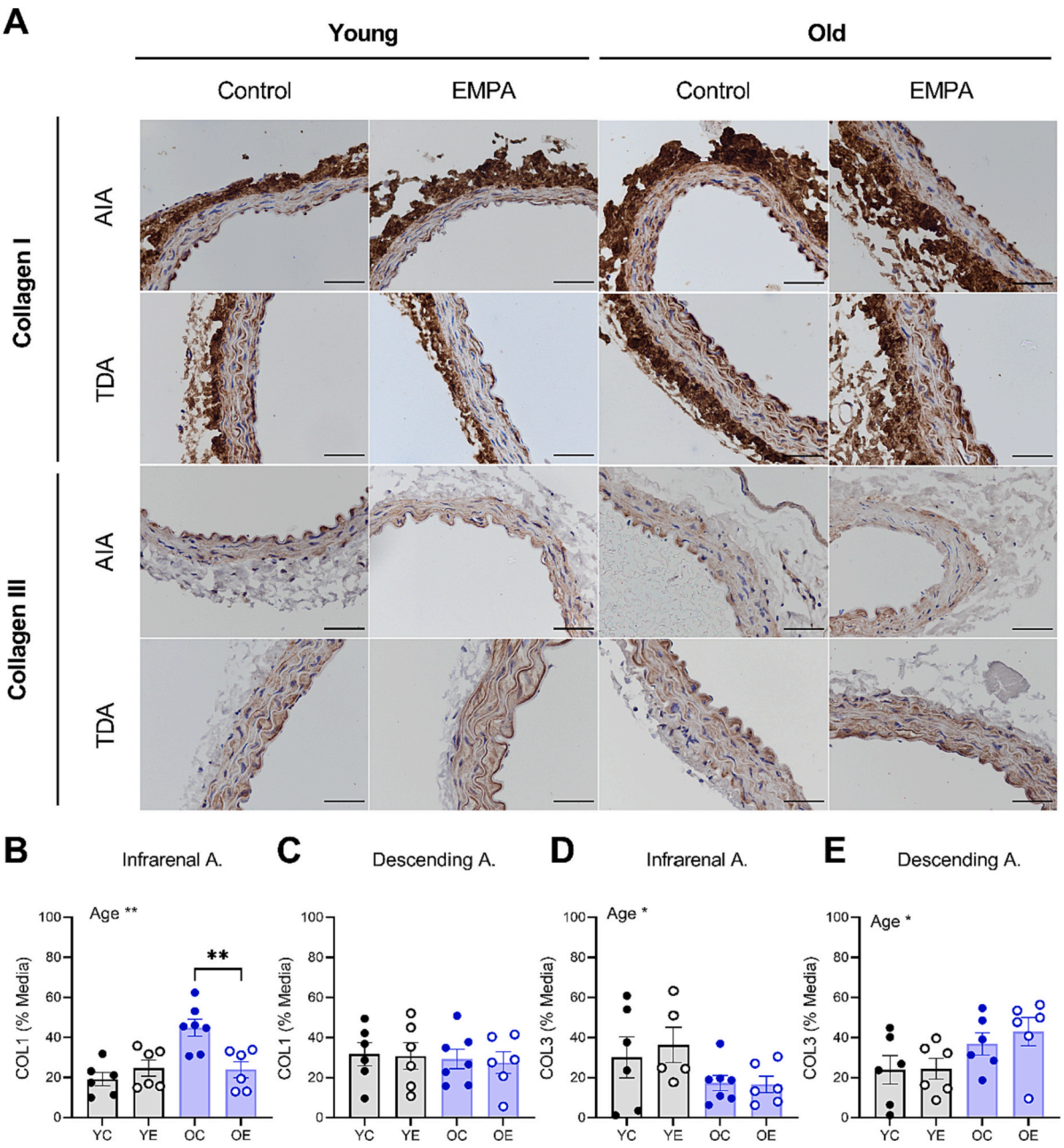

Fig. 4. Empagliflozin decreases collagen type I in the tunica media of the abdominal infrarenal aorta, but not thoracic descending aorta. Immunohistochemical stains for collagen type I and type III were performed to assess ageing-induced aortic fibrosis and whether empagliflozin attenuates collagen deposition. (A) Representative images of young and old aortic tissue stained with anti-collagen I or anti-collagen III. Scale bar = 50 μm. (B) Quantification of medial collagen deposition (as % area = (area collagen/area medial layer)*100) revealed a significant increase in collagen type I in the infrarenal aorta of old mice. Furthermore, empagliflozin treatment significantly decreased collagen type I in the medial layer of the infrarenal aorta of old mice. (C) There were no differences in collagen type I in the thoracic descending aorta. (D, E) Medial collagen type III content was significantly altered in aged mice. Surprisingly, whereas collagen type III was decreased in the aged abdominal infrarenal aorta, an increase was observed in the aged thoracic descending aorta. However, these age-related changes were unaffected by empagliflozin treatment. $n = 6$ for YC, YE and OE, $n = 7$ for OC. Statistical analysis was performed by using a Two-Way ANOVA with Sidak *post hoc* test for multiple comparisons. ** = $p < 0.01$, *** = $p < 0.001$. **TDA** = Thoracic Descending Aorta; **AIA** = Abdominal Infrarenal Aorta; **YC** = Young mice, control; **YE** = Young mice + empagliflozin; **OC** = Old mice, control; **OE** = Old mice + empagliflozin.

observed in the medial layer of the aged TDA (Fig. 4, D, E). However, age-related changes in collagen type III content in the medial wall were not affected by empagliflozin treatment. Further, neither ageing nor empagliflozin treatment altered collagen type I and type III levels in the thoracic ascending aorta (Supplementary Fig. 1).

3.6. Empagliflozin reduces aortic TGF-β in the infrarenal, but not the thoracic descending aorta of aged mice

An immunohistochemical staining for TGF-β was performed on aortic segments from young and old mice which were treated with or without empagliflozin (Fig. 5, A). There were no changes in the amount of medial TGF-β in the TDA between the different groups (Fig. 5, B). Furthermore, ageing tended to increase medial TGF-β content in the

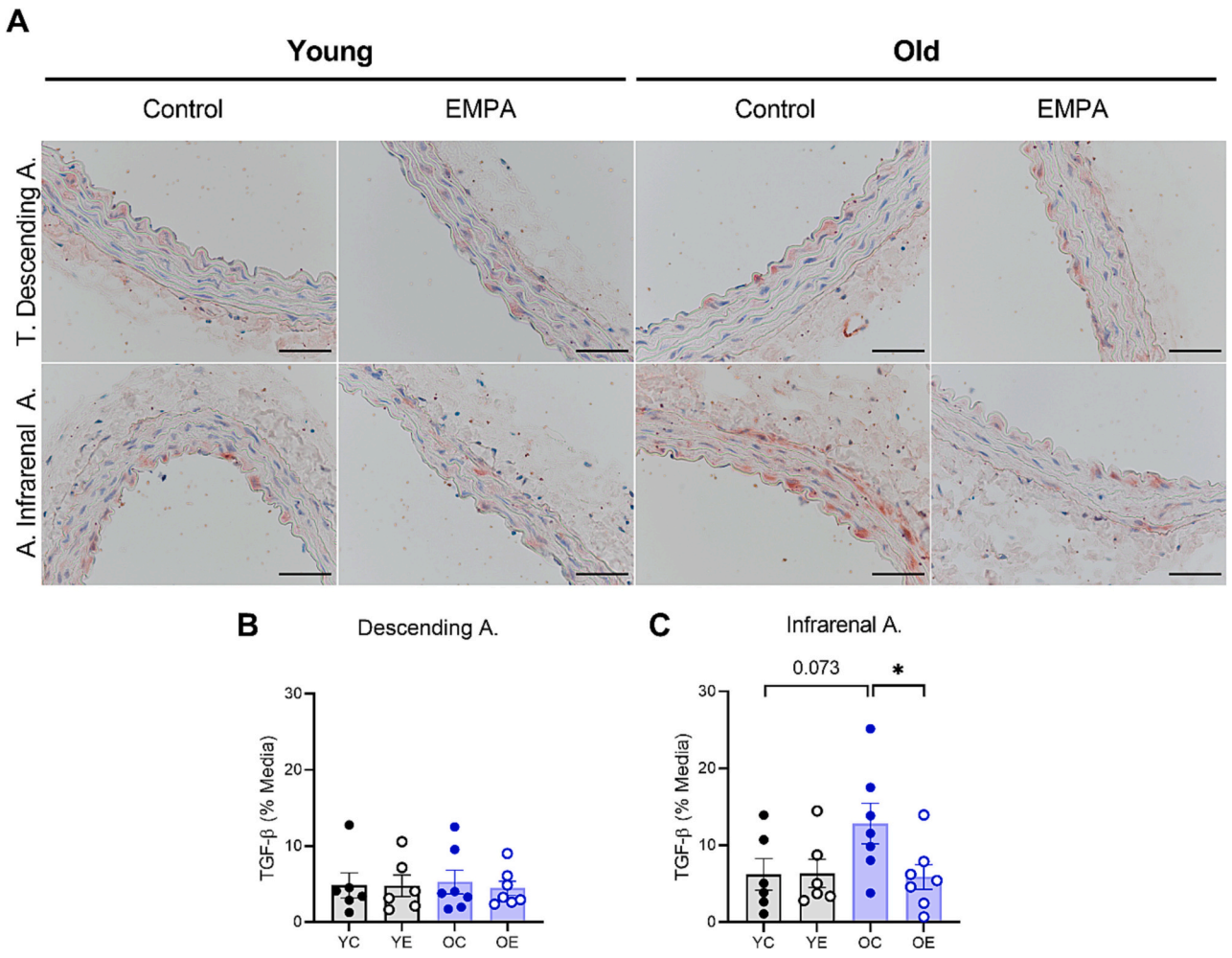

Fig. 5. Empagliflozin decreases aortic medial TGF-β in the aged infrarenal aorta. An immunohistochemical staining for TGF-β was performed on aortic segments from young and old mice (with or without empagliflozin (15 mg/kg/day) treatment) after they were subjected to biomechanical testing. (A) Representative images of aortic tissue stained for TGF-β. (B, C) There were no changes in medial TGF-β in the thoracic descending aorta between the different groups, nor did ageing significantly affect the amount of TGF-β in the medial layer of the AIA of old mice ($p = 0.073$). (C) Alternatively, empagliflozin treatment in old mice significantly decreased ($p = 0.047$) medial TGF-β in the AIA. Scale bar = 50 μm. n = 6 for YC and YE, n = 7 for OC and OE. Statistical analyses: Two-Way ANOVA with a Sidak post-hoc test for multiple comparisons. **T. Descending A.** = Thoracic Descending Aorta; **A. Infrarenal A.** = Abdominal Infrarenal Aorta; **YC** = Young mice, control; **YE** = Young mice + empagliflozin; **OC** = Old mice, control; **OE** = Old mice + empagliflozin.

abdominal infrarenal aorta, yet not reaching statistical significance (Fig. 5, C). Interestingly, empagliflozin treatment in old mice significantly decreased medial TGF-β in the abdominal infrarenal aorta (Fig. 5, C).

3.7. Empagliflozin attenuates TGF-β induced vascular smooth muscle cell fibrosis in vitro

TGF-β (10 ng/mL) stimulation of primary murine aortic VSMCs significantly increased collagen production, demonstrated as an increase in Sirius red positivity (Fig. 6). Interestingly, treatment of VSMCs with 50 μM empagliflozin combined with 10 ng/mL TGF-β did not significantly increase collagen production compared to the control. Furthermore, empagliflozin treated cells had a significantly lower collagen deposition compared to the TGF-β treated cells ($p = 0.0032$) but there was no difference compared to the TGF-β + empagliflozin treated cells.

4. Discussion

In the present study, we have demonstrated that pharmacological treatment with empagliflozin decreased central aortic stiffness in old, non-diabetic, female C57Bl6/J mice. Moreover, empagliflozin attenuated ageing-induced vascular fibrosis in the infrarenal aorta since it attenuated the ageing-associated increase in medial collagen type I and TGF-β deposition. Yet, empagliflozin was not able to prevent or reduce all age-related changes in the vasculature, such as elastin fragmentation in the ascending aorta and altered VSMC contractility.

There are contradictory findings in literature regarding the effect of empagliflozin on arterial stiffness in patients. Whereas some studies have shown that empagliflozin reduced arterial stiffness in patients with type 1 or type 2 diabetes mellitus, others have reported no change in pulse wave velocity (PWV), a measure of arterial stiffness [27–31].

The present study shows that 7 weeks of *in vivo* empagliflozin treatment decreases arterial stiffness of the aorta in old normoglycemic mice, measured *ex vivo* as the relationship between the Peterson's modulus and pressure (which is similar to the classic "stress-strain" relationship). These results are in agreement with findings from other studies with *ex vivo* biomechanical evaluation of (central) blood vessels. Empagliflozin has been shown to decrease the stiffness of the renal arteries of female, hyperglycaemic, *db/db* mice after 5 weeks of treatment [32]. Another study demonstrated the reversal of ageing-induced arterial stiffness, both *in vivo* (through PWV) and *ex vivo*, of both the mesenteric arteries and the thoracic aorta after 6 weeks of empagliflozin treatment in aged male mice [33].

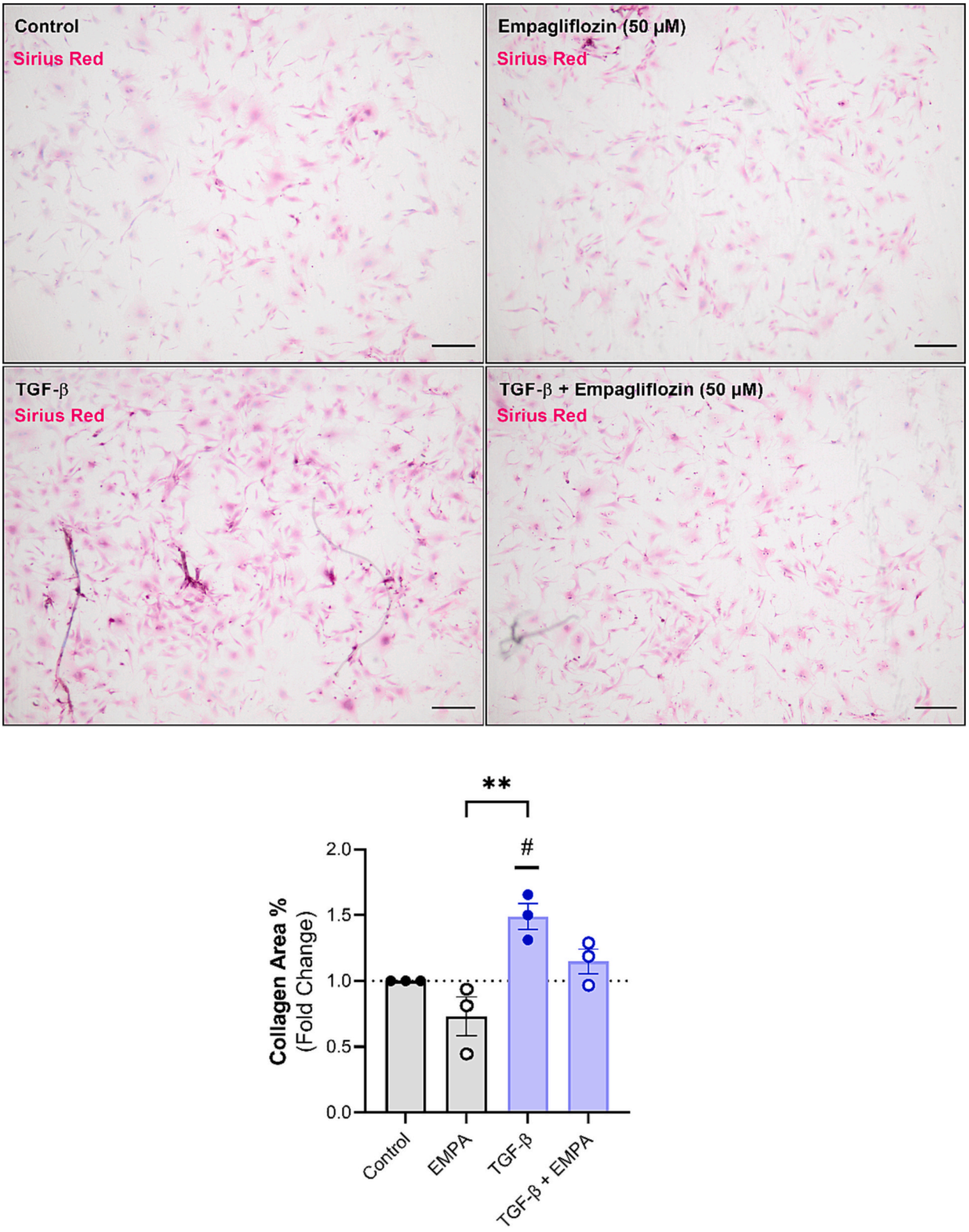

Fig. 6. Empagliflozin attenuates TGF-β induced collagen production in murine primary vascular smooth muscle cells. Collagen production was induced in primary murine aortic vascular smooth muscle cells (mVSMCs) with TGF-β. Collagen was visualized by performing a Sirius Red stain. Stimulating VSMCs with TGF-β induced collagen production, as demonstrated by a significantly ($p < 0.05$) increased Sirius Red area (%area, normalized against number of cells per image). Interestingly, treating VSMCs with both 50 μM empagliflozin and 10 ng/mL TGF-β did not result in an increased amount of collagen content. Scale bar = 250 μm. $n = 3$. Statistical analyses: one-sample *t*-test (hypothetical value = 0) on Log_2 transformed fold change data to determine statistical significance *versus* control; $\#p < 0.05$. An additional One-Way ANOVA with a Tukey *post hoc* test for multiple comparisons was performed to compare collagen deposition between the "EMPA", "TGF-β" and the "TGF-β + EMPA" groups. ** = $p < 0.01$. **EMPA** = Empagliflozin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We demonstrated that 7 weeks of empagliflozin treatment decreases the *ex vivo* stiffness of the thoracic descending aorta and the abdominal infrarenal aorta in aged, normoglycemic, female mice. Furthermore, an increased medial wall thickness was observed in the abdominal infrarenal aorta from old mice when treated with empagliflozin. However, there was no difference in diameter of the abdominal infrarenal aorta between old empagliflozin-treated and untreated mice. According to the law of Laplace, an increase in wall thickness (at an equal pressure and radius) reduces wall stress. Hence, the increased wall thickness could be an explanation for the attenuation of the pressure-Ep relationship after empagliflozin treatment. It is thus far unclear how empagliflozin increased medial wall thickness of the abdominal infrarenal aorta, warranting further research. Taken together, the present study provides complementary results to existing studies that reported attenuation of arterial stiffness by empagliflozin, independent of blood glucose levels.

Whereas empagliflozin decreased the stiffness of both the thoracic descending aorta and the abdominal infrarenal aorta, it did not decrease ageing-associated arterial stiffness in the thoracic ascending aorta. It is known that aortic elastin becomes fragmented with ageing, altering the biomechanical properties of the aorta [1,34,35]. Indeed, while elastin fragmentation was observed in the ascending aorta of aged mice, 7 weeks of treatment with empagliflozin did not prevent or decrease elastin fragmentation. We, and others, have previously shown that elastin fragmentation is detrimental to vascular function, shifting the pressure load to the stiffer collagen fibres, and increasing the functional stiffness of the blood vessel [36–38]. Accordingly, ageing-induced elastin fragmentation in the ascending aorta directly contributes to the increased aortic stiffness that was observed *ex vivo*. It should be noted that ageing-induced elastin fragmentation was only observed in the ascending aorta and not in the thoracic descending aorta or in the abdominal infrarenal aorta. We therefore speculate that loss of functional elastin fibres impedes the beneficial effects of empagliflozin on the biomechanical properties of the ageing thoracic ascending aorta.

Old mice had a significantly higher amount of collagen type I in the medial layer of the abdominal infrarenal aorta, whereas no age-related changes in collagen deposition in the medial layer of the thoracic descending aorta were observed. Concomitantly, there was an increased amount of TGF-β in the medial layer of the abdominal infrarenal aorta. Short term (7 weeks) empagliflozin treatment reduced ageing-induced changes in both medial collagen type I and medial TGF-β deposition in the abdominal infrarenal aorta. This finding is in line with other studies highlighting the antifibrotic effect of SGLT2 inhibition on the cardiovascular system. Indeed, empagliflozin inhibits the TGF-β/Smad pathway, ameliorating both diabetes-associated myocardial fibrosis and peritoneal fibrosis [39,40]. Moreover, TGF-β/Smad inhibition by dapagliflozin attenuates myocardial fibrosis in normoglycemic rats with heart failure [41]. In concordance with these studies, we demonstrated that empagliflozin (50 μM) attenuates TGF-β-induced collagen production in primary VSMCs. This finding indicates a direct anti-fibrotic effect of empagliflozin on VSMCs. Indeed, since SGLT2 is expressed on vascular cells, the anti-fibrotic effect of empagliflozin on ageing-induced vascular fibrosis could be a result of direct vascular SGLT2 inhibition [21].

However, the empagliflozin-induced reduction of both medial TGF-β and medial collagen do not fully explain its attenuation of age-related arterial stiffening. In the present study, empagliflozin decreased the *ex vivo* stiffness of the thoracic descending aorta in the absence of (anti-) fibrotic effects. Medial collagen or TGF-β deposition were not increased in the aged thoracic descending aorta. Therefore, a reduction of collagen or TGF-β, as seen in the abdominal infrarenal aorta, does not explain the decrease in arterial stiffness of the thoracic descending aorta. Empagliflozin treatment either acts differently on the thoracic descending aorta than it does on the abdominal infrarenal aorta, or it acts on a different mechanism that affects both types of aortic tissue. For example, the degree of collagen or elastin cross-linking was not measured in the present study. The amount of collagen cross-links increase with age and is a major contributor to the development of arterial stiffness [42,43]. It has been shown that there is an increased deposition of advanced glycation end products (AGEs), which are potent cross-linkers of collagen, in the thoracic aortic wall of old mice [44,45].

SGLT2 inhibition has been demonstrated to suppress the AGEs-RAGE axis, whereas empagliflozin has been demonstrated to decrease the accumulation of AGEs in renal arterioles [32,46–48]. Therefore, while empagliflozin decreased ageing-associated fibrosis in the abdominal infrarenal aorta, other ageing-related changes in the vasculature (*e.g.*, deposition of AGEs) could be targeted by SGLT2 inhibition as well. Hence, the attenuation of central aortic stiffness by empagliflozin could be of multifaceted nature.

The pleiotropic effects of empagliflozin warrant further investigation. It remains unclear how empagliflozin reduces arterial stiffness and whether this is because of a direct involvement of SGLT2 in the vasculature. Whereas direct effects of SGLT2-inhibitors on the vasculature have been demonstrated, such as decreasing endothelial cell activation, it cannot be excluded that systemic effects of empagliflozin, such as a decrease in systemic inflammation, could also beneficially affect the vasculature [49,50]. Indeed, we have shown that aged, female, mice have bigger spleens than young mice. It has been previously shown that spleen weight increases with age in mice, with concomitant changes in splenic immune cell profiles and circulating cytokines [51]. Since empagliflozin treatment in old female mice significantly decreased spleen weight, an effect on the immune system cannot be excluded and warrants further research.

In conclusion, empagliflozin (and perhaps other SGLT2-inhibitors as well) seems to be a promising therapeutic agent to treat arterial stiffness in normoglycemic conditions, yet its mechanism of action on the aorta is not fully elucidated. Future studies should explore the physiological role of SGLT2 (inhibition) in the vasculature and its role in ageing to gain a better understanding of the use of pharmacological agents such as empagliflozin as treatment options for vascular ageing.

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CRediT authorship contribution statement

Cédric H.G. Neutel: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Callan D. Wesley:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Melissa Van Praet:** Formal analysis, Investigation, Writing – review & editing. **Celine Civati:** Investigation. **Lynn Roth:** Writing – review & editing, Supervision. **Guido R.Y. De Meyer:** Conceptualization, Writing – review & editing. **Wim Martinet:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Pieter-Jan Guns:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2023.107212.

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